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Trophic connectivity in coastal habitats supporting fishery species

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This dissertation is submitted to the University of Algarve,

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Declaração da autoria do trabalho

Tese: Trophic connectivity in coastal habitats supporting fishery species

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluídas.

Apoio

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To Mafalda and Lucas,

mission I'Mpossible

Abstract

The transfer of production along coastal habitats from migration of organisms and natural or anthropogenically caused environmental conditions create complex food webs between habitats.

A species is rarely independent from the resources of other habitats as nutrients and food supply flow easily in the aquatic environment developing strong habitat connections between food webs. These multiple trophic interactions are very susceptible to variability in the environmental conditions of the habitat that supplies the energy source. All this justifies additional investigation on coastal habitat specific food webs interactions and key energetic connections that extent from primary producers to top consumers.

Given the easy access and importance to achieve sustainability of species and their supporting habitats, explicitly for fisheries species that maintain local economy, this thesis aims to study energy sources, prey and consumers that transcend habitats in the eastern Algarve coastal area.

A General Introduction highlights the functioning of coastal food webs and the flow of nutrients and food with the movement of organisms between distinct habitats in the coastal area. The constant changes in the coastal landscape and the variability in environmental conditions create a knowledge gap in the functional role of different habitats in the complex web of coastal trophic interactions and secondary production that support coastal fisheries. Temporal and spatial variability in trophodynamics of fisheries species was performed with trophic markers: fatty acids and stable isotopes. These biomarkers determine the importance of the outwelling of terrestrial organic

matter in trophic webs of adjacent coastal habitats and if movement of consumers results in the transfer of energy sources between habitats.

This introduction describes the most relevant aspects in trophic markers biochemistry and applications in ecology and detailed analytical procedures for the following chapters, as well as quality control of analysis. Applications in ecological studies are exemplified from the identification of energy sources, including terrestrial organic matter, microphytobenthos and different groups of phytoplankton that support the food webs of a wide variety of aquatic consumers.

Chapter 2 demonstrates how the wide range of primary producers and energy sources is reflected in the diet of coastal fish, estuarine fish and offshore fish. The relative contribution of energy sources determined the importance of upriver foraging behavior of marine fish for the transport of marine production upriver and the importance of the estuarine environment as feeding ground for coastal fishes. Estuary primary producers and vascular plants were recognized as a potential source of energy for mid estuary fishes against marine phytoplankton and microphytobenthos for coastal fishes.

Chapter 3 describes the role of terrestrial organic matter as energetic source for fish food webs in the estuary and adjacent coastal area. Organic detrital material of terrestrial origin flows in rivers towards coastal areas in a dynamic and continuous process but nevertheless it had a minor role in this connectivity between the estuarine and coastal trophic webs.

Chapter 4 evaluates several tissues of a cephalopod from the benthic coastal environment to define inter-tissue comparisons. Tissues with the same function represented the trophic markers in similar proportions and were ineffective to determine temporal and spatial diet variability. Mantle and digestive gland were the chosen tissues for the sequent analyses.

Chapter 5 demonstrates the main sources of nutrition in the diet of *Octopus vulgaris* from the coastal benthic environment. Here are described potential trophic pathways to other habitats, including the role of terrestrial production. This chapter explains the

temporal variability in the contribution of the different production sources for the octopus food web in the area of the fishery and demonstrates octopuses depend mainly on local primary sources. The trophic markers supported a trophic web based in dinoflagellate production in the marine environment.

Chapter 6 shows the feeding behavior of the cephalopod *O. vulgaris* towards the input of allochthonous prey and how human interactions create a trophic coupling between the benthic and pelagic environment. The opportunistic behavior of this cephalopod was demonstrated for a food resource absent from the natural diet.

Chapter 7 is the general discussion from the overall results and conclusions of the case studies in this thesis under the subject of habitat trophic connectivity of coastal fishery species. Pros and cons of the chosen techniques are discussed as well as issues arising from the sample sizes and pooling of samples. Further research and techniques will improve studies on trophic dynamics and connectivity in these ecologically and economically important benthic habitats.

Keywords: coastal benthic trophic web, estuary, fish, *Octopus vulgaris*, diet variability, trophic markers, fatty acids, stable isotopes, terrestrial organic matter, allochthonous energy sources, primary producers.

Sumário estendido

A transferência de produtividade ao longo de habitats costeiros através da migração de organismos e das condições ambientais naturais ou de origem antropogénica, desenvolvem redes tróficas complexas entre habitats.

Uma espécie é raramente independente dos recursos de outros habitats, pois o fluxo de nutrientes e alimento é constante no meio aquático o que origina fortes ligações entre redes tróficas. Estas múltiplas interações tróficas são suscetíveis à variabilidade nas condições ambientais do habitat que providencia a fonte energética. Estes factos justificam um maior desenvolvimento na investigação de interação entre redes tróficas de diferentes habitats costeiros e de ligações energéticas chave com efeito dos produtores primários até aos consumidores de topo.

O acesso fácil e a elevada importância para a sustentabilidade das espécies e dos seus habitats, especificamente devido à economia pesqueira local, fundamentam esta tese sobre as fontes de energia, presas e consumidores que ultrapassam diversos habitats costeiros na região este do Algarve.

Um Introdução Geral explica o funcionamento das redes tróficas costeiras no âmbito do fluxo de nutrientes e alimento com o movimento de organismos entre habitats distintos ao longo da zona costeira. As constantes alterações na paisagem e a variabilidade das condições ambientais provocam uma lacuna no conhecimento acerca dos diferentes habitats na rede complexa de interações tróficas na zona costeira e na produção secundária que sustenta as pescas costeiras. A variabilidade temporal e espacial na dinâmica trófica de espécies piscatórias foi realizada com marcadores tróficos: ácidos gordos e isótopos estáveis. Estes biomarcadores determinam a importância da exportação de matéria orgânica terrestre através dos rios em habitas costeiros e determinam se o movimento de consumidores resulta na transferência de energia entre habitats.

Esta introdução descreve os aspetos mais relevantes na bioquímica dos marcadores tróficos e exemplifica a aplicabilidade em ecologia e os procedimentos analíticos

detalhados para os capítulos seguintes, tal como o controlo de qualidade nas análises. São exemplificados estudos de fontes energéticas, incluindo de origem terrestre, microfitobentos e diversos grupos de fitoplâncton que sustentam cadeias tróficas de vários consumidores aquáticos.

O capítulo 2 demonstra como uma grande variedade de produtores primários e fontes energéticas é refletida na dieta de peixes costeiros e estuarinos. A contribuição relativa das fontes de energia nos peixes no estuário determinou a importância do movimento dos peixes costeiros para montante no transporte da produção marinha para o estuário e da importância do estuário como local de alimentação. Produtores primários estuarinos e plantas vasculares foram reconhecidos como fontes de energia para a cadeia trófica dos peixes no estuário médio, enquanto fitoplâncton marinho e microfitobentos foram as fontes energéticas mais importantes para os peixes no baixo estuário e zona costeira.

O capítulo 3 descreve o papel da matéria orgânica de origem terrestre como fonte energética para peixes no estuário e na zona costeira adjacente. Os detritos de origem terrestre fluem nos rios em direção ao mar num processo dinâmico e contínuo mas no entanto o seu papel foi secundário na conectividade entre as redes tróficas de peixes na zona estuarina e costeira.

O capítulo 4 avalia diversos tecidos de um cefalópode da zona bentónica costeira com o objetivo de definir comparações sequentes entre tecidos. Tecidos com a mesma função representaram marcadores tróficos em proporções similares e a sua comparação é ineficaz para determinar variabilidade na dieta na escala temporal e espacial.

O capítulo 5 demonstra as principais fontes de nutrição na dieta do *Octopus vulgaris* da zona bentónica costeira. Aqui são descritas potenciais ligações tróficas a outros habitats, incluindo a importância de matéria orgânica de produção terrestre. Este capítulo explica a variabilidade temporal da contribuição de fontes de energia com origem distinta para a rede trófica do polvo na área da sua pesca e demonstra que os polvos dependem principalmente de fontes de produção primária locais. Os

marcadores tróficos confirmam que esta rede trófica tem como base dinoflagelados marinhos.

O capítulo 6 mostra o comportamento alimentar do polvo *O. vulgaris* em relação à importação de alimento de um ambiente distinto e o potencial das atividades humanas para criar ligações tróficas entre o ambiente pelágico e bentónico. O comportamento oportunístico alimentar deste cefalópode foi demonstrado pela facilidade com que ingeriu uma presa ausente da sua dieta natural.

O capítulo 7 é uma discussão geral dos resultados principais e das conclusões dos estudos efetuados nesta tese sob o tema da conetividade trófica de habitats de espécies costeiras com interesse piscatório. Pros e contras das técnicas utilizadas são discutidas, tal como questões derivadas das amostragens, número e *pool* de amostras. Novas investigações e técnicas melhoram os estudos de dinâmica trófica e das ligações tróficas entre habitats de espécies importantes em termos ecológicos e económicos

Palavras-chave: rede trófica bentónica costeira, estuário, peixe, *Octopus vulgaris*, variabilidade na dieta, marcadores tróficos, ácidos gordos, isótopos estáveis, matéria orgânica terrestre, fontes energéticas alóctones, produtores primários.

Thesis structure

This PhD thesis consists of a general introduction on the importance of energy sources to connect habitats of several coastal fishery species and include the description of the trophic markers and the techniques chosen for the analyses. Two chapters follow this introduction with the objectives to determine the main sources of energy for fishes in an estuary and adjacent coastal waters, connectivity between these habitats and food dependence on local resources.

Chapter 2 determines the base of the food web of fishes in the estuary and the importance of movement of fish in the estuary transfer of energy and habitat connectivity between the estuarine and coastal environment. Chapter 3 discusses the importance of an abundant source of organic matter - terrestrial organic matter – on the trophic web of fishes in the estuary and coastal waters and how the terrestrial environment is connected to the estuarine and marine environment through the fish food web.

Next, an important species for the coastal fisheries, *Octopus vulgaris* is studied in three chapters for diet variability, energy sources for the trophic web and influence of human activities in food supply and potential effects on distant habitat connectivity.

Chapter 4 and Chapter 5 aim to evaluate tissue comparison to study diet variability and determine diet shifts and variability at the base of the trophic web. Allochthonous sources of primary producers will indicate a trophic connectivity to other habitats.

Chapter 6 demonstrates how an artificial link between distant habitats is created by human activities. The opportunistic feeding behavior of the cephalopod is explored by the use of allochthonous prey that creates a distant habitat coupling. The immediate consumption of this type of prey despite the unavailability in the natural diet supports the feeding behavior characteristic of this species.

The last chapter of this thesis, the General Discussion, shows how trophic webs in several species in coastal ecosystems are affected by diet variability and sources of

energy at the base of the trophic web and the potential of environmental conditions and anthropogenic activities on trophic connectivity between habitats. The analyses of these diet adjustments to environmental conditions or new supply of food are quickly obtained by the combination of trophic markers and inter-tissue comparisons. This purpose to define important trophic links between habitats of fisheries species will result on predictions of species sustainability and coastal productivity.

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1 General Introduction

Coastal ecosystems have typically diverse habitats with functional roles that include feeding and protection for species. High productivity and biodiversity is strongly dependent on landscape and environmental conditions for the production and flow of energy, community structure and trophic dynamics (Abrantes et al. 2015).

The multiple trophic interactions between nutrients, food and consumers within and across habitats affect the whole ecosystem (Abrantes et al. 2015) and the production and origin of energy with distinct quality and quantity from habitats with different functional roles are still not well understood for the trophic dynamics of coastal fishery species. Habitat trophic connectivity depends on the distance between habitats, relative productivity, mobility of consumers (Polis et al. 1997), prey availability, life strategy of organisms (Doucett et al. 1996, Kimmerer 2002, Vinagre et al. 2008) and foraging behavior across habitats (Kirsch et al. 1998a).

The complex food webs that connect the pelagic-benthic and land-ocean environments and their dynamics is highly coupled to spatial and temporal variability in environmental conditions and energy sources and require research (Budge et al. 2002, Deudero et al. 2004, Sherwood and Rose 2005, Carlier et al. 2007, Petursdottir et al. 2008, Polis and Strong 2009) to set priorities in habitat conservation and rehabilitation and coastal fisheries management (Doucett et al. 1996, Kirsch et al. 1998, Kimmerer 2002, Vinagre et al. 2008).

Nutrient input is a major factor in trophic webs of coastal areas to stimulate phytoplankton production and define the dominant group that fuels the food web. Any modification in the nutrients supplied to primary producers is transferred to higher trophic levels and ultimately affects secondary productivity and biodiversity. The input of particulate organic material derived from land or estuaries to the coastal benthic environment may greatly exceed *in situ* production to support the community (Polis et

al. 1997, Riera and Richard 1997, Kang et al. 2008, Schlacher et al. 2008, Kostecki et al. 2010) and is strongly affected by inland or coastal human activities (Connolly et al. 2005b, 2009, Darnaude 2005). Terrestrial material affects the supply and flow of nutrients and may have cascading effects on trophic interactions (Deudero et al. 2004).

Microalgae, bacteria and macroalgae are the main primary producers with diatoms and dinoflagellates as the major contributors of energy to higher trophic levels via consumption by copepods, larvae and other invertebrates (Riera et al. 1999, Rossi et al. 2006, El-Sabaawi et al. 2009, Lebreton et al. 2011).

Foraging behavior of consumers between habitats and the input of allochthonous prey creates links between distant trophic webs. Migration of coastal fishes upriver link the marine and estuarine environment with possible effects on estuarine key species (Polis et al. 1997) and pelagic organisms used as bait in coastal benthic fisheries may link the pelagic and benthic environments with effects on the predator population and community structure.

A range of methodologies are applied for the study of trophodynamics. Stomach (gut) contents analyses provides short term dietary information but the miscalculation for the contribution of food sources, mainly organisms without hard parts and easily digested is considerable (Sherwood and Rose 2005, Carlier et al. 2007, Petursdottir et al. 2008). The physiological responses from the adaptation of an organism to changes in the food web are explored by the analysis of chemical compounds designated as trophic markers.

Trophic markers accumulate in the tissues of consumers over a certain period of time and reflect those of their food source. This feature allows to determine the pathway of energy through multiple trophic compartments, the primary producers for the food web (Budge et al. 2002, Post 2002, Deudero et al. 2004, Sherwood and Rose 2005, Alfaro 2006, Carlier et al. 2007, Petursdottir et al. 2008) and trophic shifts through inter-tissue comparison (MacNeil et al. 2005, Stowasser et al. 2006).

Fatty acids and stable isotopes, $\delta^{15}N$ and $\delta^{13}C$, are commonly used trophic markers and complementary for the analysis of energetic sources to the food web and diet

variability of consumers (Iverson et al. 2002, Estrada et al. 2005, Melville and Connolly 2005, Budge et al. 2006, Falk-Petersen et al. 2009, Hussey et al. 2011, Stowasser et al. 2012).

The fatty acids and the stable isotopes are quasi perfect trophic markers. A perfect trophic marker is a compound with easily identifiable origin, inert, not selectively processed during food uptake and incorporation, and transferred from one trophic level to the next in a predictable manner (Dalsgaard et al. 2003, de Lange and Van den Brink 2006).

This study focuses on trophic connectivity in benthic coastal habitats and in temporal and spatial variability of sources of energy to species relevant for local fisheries with different features in foraging behavior and supply of food sources. Coastal fishes move across habitats and potentially transfer production through foraging behavior, whereas other benthic species, like the cephalopod *Octopus vulgaris*, occupies a discrete habitat that provides the resources necessary for the benthic life stage. The thesis aims to answer four main questions: 1) Does foraging behavior of coastal fishes connects the marine and estuarine trophic webs?; 2) What is the importance of the contribution of terrestrial organic matter for coastal fish food webs?; 3) Is there a temporal variability in the exploitation of the resources of the benthic environment from a resident benthic species that avoids competition with conspecifics?; 4) Is the input of an allochthonous prey a potential link between habitats?

These questions are answered in five chapters. Firstly the focus is on some of the most common coastal fish in a habitat that connects the terrestrial and marine environments, an estuary. This is an opportunity to investigate the dynamics of specific trophic connectivity between habitats with different functional roles during spring and early summer and it is expected a recent diet with stronger contribution of estuarine and terrestrial production sources if the movement of fishes to the estuary has feeding purposes.

Then, the study focuses on the variability and importance of energetic sources that sustain the productivity of a coastal benthic cephalopod, in the late spring. The main

question is on the dietary choices over time of this species. The final case analyzed the opportunistic feeding behavior of this cephalopod towards an allochthonous food source used as bait to benefit fisheries and that is expected to connect energetically different environments if fishing effort increases the amount of input of this bait.

This thesis ends with the discussion of the improvements required for future research in dietary analysis variability using trophic markers and with the discussion of the importance to acknowledge habitat connectivity through variability in prey and energy sources, especially for fisheries species that transcend habitats.

1.1 Trophic markers

1.1.1 Fatty acids

1.1.1.1 Introduction

Fatty acids composition of organic material detail consumer-food relationships. For example, the link of zooplankton, where microalgae are the principal primary producers, to higher order consumers is detected if patterns in fatty acids composition are incorporated and transferred in a conservative way. Organic matter assimilated by organisms from non-living sources, e.g. terrestrial matter, is also detected from the characteristic pattern of the fatty acid composition (Dalsgaard et al. 2003).

1.1.1.2 Fatty acid nomenclature and biochemistry

The abbreviations follow conventional nomenclature (IUPAC-IUB): in the format X:Y(n-z), X refers to the chain length (number of carbon atoms, including the carboxylic acid or alpha carbon), Y refers to the number of carbon-carbon double bonds and z refers to the position of the first carbon-carbon double bond in the molecule relative to the terminal methyl group (carbon number 1 in the n-z system, i.e. omega carbon) (Table 1.1.1).

Table 1.1.1 Nomenclature of the most representative fatty acids in lipids of fish muscle and liver.

Systematic Name	Trivial Name	Abbreviation
Saturated		
tetradecanoic acid	myristic acid	14:0
pentadecanoic acid		15:0
hexadecanoic acid	palmitic acid	16:0
heptadecanoic acid	margaric acid	17:0
octadecanoic acid	stearic acid	18:0
eicosanoic acid	arachidic	20:0
Monounsaturated		
cis-9-hexadecenoic acid	palmitoleic acid	16:1 (n-7)
cis-9-octadecenoic acid	oleic acid	18:1 (n-9)
cis-11-octadecenoic acid	asclepic acid or cis-vaccenic acid	18:1 (n-7)
cis-9-eicosenoic acid	gadoleic acid	20:1 (n-11)
cis-11-eicosenoic acid	gondoic acid	20:1 (n-9)
cis-11-docosenoic acid	cetoleic acid	22:1 (n-11)
cis-13-docosenoic acid	erucic acid	22:1 (n-9)
cis-15-tetraeicosenoic acid	nervonic acid	24:1 (n-9)
Polyunsaturated		
hexadecadienoic acid		16:2
cis-9,12-octadecadienoic acid	linoleic acid	18:2 (n-6)
cis-11,14-eicosadienoic acid		20:2 (n-6)
hexadecatrienoic acid		16:3
cis-6,9,12-octadecatrienoic acid	γ-linolenic acid	18:3 (n-6)
cis-9,12,15-octadecatrienoic acid	α-linolenic acid	18:3 (n-3)
cis-11,14,17-eicosatrienoic acid		20:3 (n-3)
hexadecatetraenoic acid		16:4
cis-6,9,12,15-octadecatetraenoic acid	stearidonic acid	18:4 (n-3)
cis-5,8,11,14-eicosatetraenoic acid	arachidonic acid	20:4 (n-6)
cis-8,11,14,17-eicosatetraenoic acid		20:4 (n-3)
cis-5,8,11,14,17-eicosapentaenoic acid	timnodonic acid; EPA	20:5 (n-3)
cis-6,9,12,15,18-heneicosapentaenoic acid		21:5 (n-3)
cis-7,10,13,16,19-docosapentaenoic acid	clupanodonic acid	22:5 (n-3)
cis-4,7,10,13,16,19-docosahexaenoic acid	DHA	22:6 (n-3)

Fatty acids are the main constituents of acyl lipids, such as triacylglycerols, glycolipids and phospholipids. Algae are the main primary produces of fatty acids in aquatic food webs with 15 to 20 major fatty acids. When ingested by consumers, the fatty acids are released from the backbone molecule (glycerol in the case of triacylglycerols) during digestion and enter the circulation of the consumer intact as free fatty acids. In general, these free fatty acids are transferred to the tissues and not transformed or metabolized. Once taken up by tissues in the consumer, fatty acids are either used for energy or re-esterified to a backbone molecule and stored in adipose tissue (Iverson et al. 2004). *De novo* biosynthesis of fatty acids is the common lipid pathway, i.e. the type I fatty acid synthetase. The major end product is palmitic acid, 16:0 and fatty acids with 14, 18 and 20 carbon atoms produced by chain elongation (i.e. acyl chains with 18 and 20 carbon atoms). Monounsaturated fatty acids, MUFA, produced by aerobic desaturation introduces a double bond between carbon 9 and 10 to form palmitoleic acid 16:1(n-7), oleic acid 18:1(n-9) and gadoleic acid 20:1(n-11). In animals, these MUFA are also biosynthesized from myristic acid 14:0 and palmitic acid 16:0 precursors obtained from diet and undergo chain elongation and desaturation. These major fatty acids end products are nowadays used to infer trophic links (Dalsgaard et al. 2003).

MUFA 20:1 and 22:1 are *de novo* biosynthesized, mainly by herbivorous calanoid copepod species and PUFA (n-3) and (n-6) are *de novo* biosynthesized by plants. Oleic acid 18:1(n-9) is the precursor of all (n-3) and (n-6) PUFA, essential to heterotrophic organisms. Linoleic acid 18:2(n-6) and α-linolenic acid 18:3(n-3) are only biosynthesized by primary producers and thus, animals only obtain these fatty acids from food.

Elongation and desaturation of 18:2(n-6) produces arachidonic acid 20:4(n-6) and elongation and desaturation of 18:3(n-3) produces EPA 20:5(n-3) and DHA 22:6(n-3). Dinoflagellates also produce 22:6(n-3) from steriadonic acid 18:4(n-3). As such, DHA is a marker for dinoflagellates dominance in aquatic environment. Diatoms have as major fatty acids: 20:5(n-3) and palmitoleic acid 16:1(n-7), from desaturation of palmitic acid 16:0 (Dalsgaard et al. 2003).

Fatty acids functional as trophic markers (FATM) provide information on energy flow in trophic webs. These fatty acids are easily identified, processed and incorporated into

consumers in a metabolically conservative manner and transferred from one trophic level to the next in a qualitative and quantitative way (Dalsgaard et al. 2003).

FATM are not species specific or metabolically stable. These compounds are affected by metabolic conditions and reproductive status of the organisms and can change predictably through the food web (Dalsgaard et al. 2003). Algae are the base of most aquatic food webs and so, the origin of most of the commonly fatty acids trophic markers. There are also bacterial and terrestrial fatty acids trophic markers. Fatty acids patterns transfer from algae to herbivores and to higher trophic levels, such as fish (Estrada et al. 2005) and marine mammals (Herman et al. 2005). In higher trophic levels, trophic relationships become unclear since fatty acids signatures come from a variety of sources. Thus, validation from other techniques is required to describe these relationships (De Lange and Van den Brink 2006).

An important aspect of FATM of the consumer is the time-integrated dietary intake. Lipid metabolism and storage in animals are organ-specific, so fatty acids extracted from specific body parts or tissues represent different feeding periods (De Lange and Van den Brink 2006).

Fatty acid trophic markers are most specific to primary producers. Microalgae have distinct fatty acid composition, for diatoms is predominant 16:1(n-7) and 20:5(n-3) and for dinoflagellates is predominant 22:6(n-3) and 18:1(n-9). Microalgae contribution to benthic organisms is detected by the proportions of each fatty acid in the tissues of consumers. The primary producers dominance in the water is detected by the ratios 16:1(n-7)/16:0 or 20:5(n-3)/22:6(n-3)>1 that indicate diatom over dinoflagellate dominance in the phytoplankton composition at the base of the trophic web. Vascular plants contribute to aquatic food webs with 18:2(n-6) and 18:3(n-3) and marine bacteria contribute with the 16:1(n-7) and 18:1(n-7). Attention is required for the whole fatty acid profile to ascertain bacterial contribution and not diatoms.

Fatty acids as EPA, DHA, ARA are essential fatty acids for animals, since they cannot synthesize them *de novo* or in sufficient amounts for metabolic requirements. The best sources of EPA and ARA are macroalgae, whereas in the seagrasses these compounds

are very scarce. The periphyton provides the best amounts of DHA (Budge and Parrish 1998, Graeve et al. 2002, Dalsgaard et al. 2003, Kharlamenko et al. 2008, Pernet et al. 2012a).

The fatty acid composition indicates the most relevant primary energy source for the food web (Graeve et al. 2002, Guest et al. 2008), e.g. cephalopods and fish have high contributions of sources rich in DHA, 22:6(n-3) (Stowasser et al. 2006, Estefanell et al. 2012a) and crustaceans have high contributions of sources rich in oleic acid 18:1(n-9) (Dalsgaard et al. 2003, Nyssen et al. 2005, Stowasser et al. 2006).

The dominant primary producers reflect nutrient availability, which in turn reflect changes in light intensity, water temperature, freshwater input, upwelling events and tidal mixing. Thus, the FATM at higher trophic levels will show any modifications in energy flow from the base of the food web associated with environmental changes, as well as variability in energetic sources between and within species due to temporal or spatial differences and ontogenetic variability in diet (MacNeil et al. 2005, Estrada et al. 2005, Petursdottir et al. 2008). The fatty acid composition is used as qualitative marker in this study.

1.1.1.3 Fatty Acids Analysis

This section details the extensive procedure for the fatty acids analysis and quality control, identical for each chapter.

1.1.1.3.1 Extraction of lipids from samples

The procedures for the lipid extraction and fatty acid analysis followed the Standard Operating Procedures of the Laboratory Manual by Marine Scotland Science and Webster et al. (2006).

1.1.1.3.2 Reagents

- Iso-hexane, methanol, dichloromethane, water, toluene; Rathburn Chemicals;

- 2,6-Di-tert-butyl- *p*-cresol butylated hydroxytoluene (BHT)]; VWR International;

- Sulphuric acid sp. Gr. 1.84, sodium chloride, potassium hydrogen carbonate (potassium bicarbonate), potassium chloride, sodium sulphate anhydrous granular; AnalaR, VWR International;

- Charcoal scrubbed nitrogen gas.

1.1.1.3.3 Reference materials

- LRM 144,LRM 147, LRM 175 (cod liver oil);

- LRM 145 (Orange Roughy oil);

- Restek Marine FAME Standard or NuCheck Standard;

- EO23;

- NuCheck.

Lipids were extracted from the fish liver and muscle; cephalopod muscle (mantle and tentacle), digestive gland and gut using a modification of the method detailed by Folch et al. (1957). Tissue samples, consisting of 1-2 g of wet weight, were removed by means of a scalpel and transferred to a clean centrifuge tube, labeled with the sample identification number, before weighing.

Samples were in chloroform/methanol (2:1 v/v; 20 fold weight of sample in volume) with 2,6-Di-tert-butyl-*p*-cresol (BHT) for at least 24h in a refrigerator (2-8^{o}C), in a centrifuge tube covered with aluminum foil.

After this time, aqueous potassium chloride (0.88% w/v) was added to form an emulsified mixture of 8:4:3 v/v/v chloroform, methanol and water. Centrifugation (1800 rpm; 15 min; 0^{o}C) was used to separate the organic and aqueous layers. The upper aqueous layer was removed and discarded into a sink using a water suction pump, connected to a Pasteur pipette. The lipid solvent mixture of the sample were transferred into clean, pre-weighted and labeled round bottom flasks using a calibrated soccorex pipette. The solid layer was air dried on a filter paper and stored frozen at -20^{o}C for stable isotopes analyses.

An aliquot of the lipid extract in solvent of several samples was transferred to screw top vials to determine lipid classes using thin layer chromatography and confirm the presence of fatty acid methyl esters, fundamental for the study. The solvent lipid mixture was evaporated completely in a rotary evaporator with a water bath set at 35^{o}C, any residual water or solvent from the lipid mixture was removed using a high vacuum pump. Each flask was weighted to determine the fraction of lipid in each tissue sample. If lipid was not visible, then 1mL of distilled toluene was added.

To determine the amount of lipid extracted from the tissue, the weight of the empty flask was subtracted from the weight of the flask with the extract. The percentage of lipid in the tissue is equivalent to the weight of lipid (g) in the tissue (g) x 100. These calculations were not always possible due to the very low weight of the lipid extract present in the flask, due to the analyzed lipid poor tissues.

The lipids extracts were trans-esterified or re-suspended in *iso*-hexane (2mL) and stored at -20^{o}C until trans-esterification.

1.1.1.4 Lipid class analysis by thin layer chromatography (TLC)

A sample of randomly chosen lipid extracts of the tissue was transferred into TLC vials for separation and identification of lipid classes. Lipid classes were separated on the basis of polarity and affinity for silica, the least polar travelling furthest along the plate.

2 µL of lipid extract in solvent was streaked onto a high performance thin layer chromatography (HP-TLC) plate and carried along the stationary phase by a mobile phase or solvent system.

The detection of the separated solutes was achieved by spraying the TLC plate with a solution of copper II sulphate in aqueous orthophosphoric acid, followed by burning.

1.1.1.4.1 Reagents

- Lipid standards mix, containing cholesterol, Cholesteryl Oleate, Oleic acid, Oleic acid methyl ester, Triolein - Sigma
- Diethyl ether - Fisher Scientific
- Copper II sulphate
- Orthophosphoric acid - BDH Limited.

1.1.1.4.2 Solvent system preparation and plate development

Three solvent systems were used to detect non polar and polar lipid classes.

Solvent system 1 was prepared with 70 mL of *iso*-hexane, 30 mL of diethyl ether; solvent system 2 was prepared with 97 mL of *iso*-hexane, 3 mL of diethyl ether; and

solvent system 3 was 100 mL of iso hexane. The solvents were poured in chromatography tanks with glass lids.

The excess gel in the TLC plate (LHP-K linear 60A high performance TLC plate, with a coating of 200 µm and size 20 x 10cm; Whatman International Ltd) was scrapped from the edges with a scalpel and allowed to dry in a fume cupboard.

On a sheet of paper with guide marks identified the lipid standards mix (edges and middle) and the samples. With a microcapillary pipette, 2µL of the lipid standard or lipid extract were transferred to the TLC layer about 0.5 cm from the top of the layer. The solvent evaporated and the samples became invisible. The plate was placed vertically into the tank with the solvent system 1 for the duration of the solvent to travel half the distance from the top. The plate was transferred to the tank with the solvent system 2 and solvent travels until 1 cm from the top. Then the plate was transferred to the tank with solvent system 3 and the solvent travelled off the top edge of the plate. The solvents were removed by evaporation after each solvent system.

The plate was developed, in a fume cupboard and upright in a aluminum foil, by spraying lightly with solution of copper II sulphate (10% w/v) in aqueous orthophosphoric acid (8% v/v), using a chromatomiser spray gun. The plate was then transferred to a pre-heated oven at 180^{o}C for 10 minutes.

Lipid classes were confirmed by comparing the Rf value (Distance lipid travelled on plate (mm)/total distance travelled of solvent on plate (mm)) with those of the standard (Figure 1.1.1). A photocopy of the TLC plates developed was kept for posterior analysis, if necessary.

The presence of fatty acid methyl esters was confirmed by the analysis of samples with the TLC procedure.

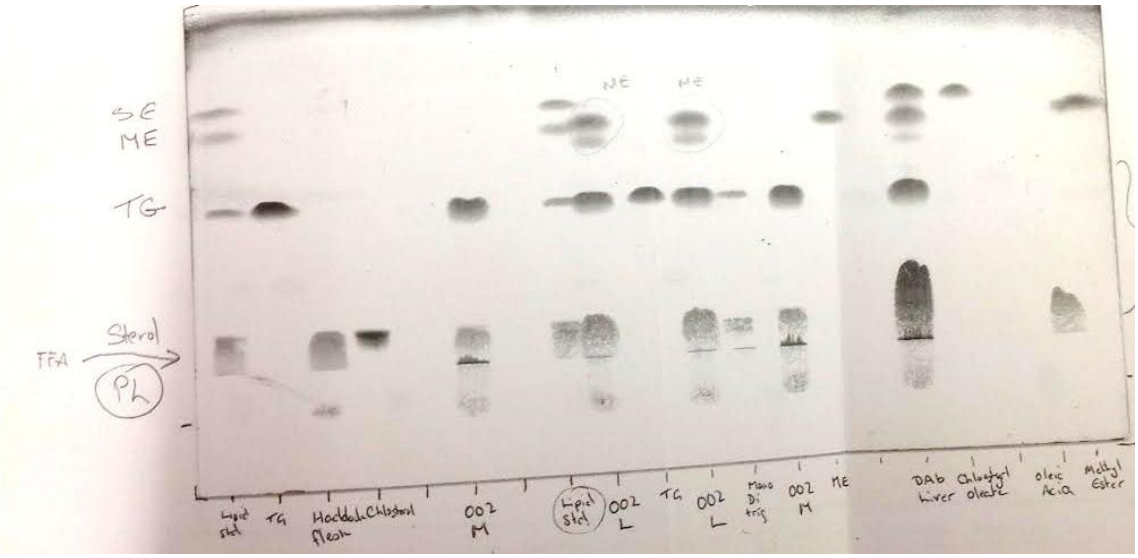

Figure 1.1.1 TLC plate developed for seabream, *Argyrosomus regius*. The presence of methyl esters is evident from the comparison of lipid classes from the solutions of other lipid standards.

1.1.1.4.3 Trans-esterification of lipids extracts from fish and cephalopod tissues

An aliquot of 10mg of the lipid extracts (or whole extract in the case of very small quantities) was transferred into 15 mL screw top test tube and 1 mL of distilled toluene was added to each tube (if not added previously as in the case of lipid poor samples).

Toluene must be distilled previously to remove residual water, which would inhibit reaction. The mixture was shaken vigorously.

Methanol-containing sulphuric acid (1% v/v; 2mL) was added to each tube and shaken. The resulting lipids were converted into fatty acid methyl esters (FAMEs) by transesterification overnight (incubated at 50°C) using a heating block.

The mixture was allowed to cool before extraction of fatty acid methyl esters with *iso*-hexane. To each tube, aqueous sodium chloride solution (5% v/v; 5mL) was added and then *iso*-hexane (2x5mL) to form an emulsified mixture; the *iso*-hexane layer (top layer) was transferred each time to a second test tube.

Aqueous potassium bicarbonate (2% w/v; 4mL) was added to the second test tube. The top layer containing the methyl esters in *iso*-hexane, were transferred to scintillation vials and dried over anhydrous sodium sulphate.

Vials were stored at -20°C until analysis, after a stream of charcoal scrubbed nitrogen. A blank and laboratory reference materials were esterified in each procedure. EO23, a laboratory reference material used to confirm results was prepared as a batch with an expiry of 1 year from the date of preparation.

1.1.1.5 Determination of fatty acids from sample tissues with GC-FID

The fatty acid methyl esters composition of the samples was determined by Gas Chromatography with flame ionization detection (GC-FID) using an Agilent (Hewlett-Packard) 5890 Series II gas chromatograph fitted with a fused silica capillary column (0.25mm i.d. x 30m) coated with a 0.25 µm film of 50% cyanopropyl. Prior to GC-FID analysis.

The extracts were diluted in *iso*-hexane to give an estimated fatty acid/ fatty alcohol concentration of 0.1 mg/mL; and 1mL of each sample was transferred to GC vials. Samples (1µL) were injected, using a cool, on-column auto injector at 60°C.

The oven temperature was ramped at 25°C/min from 60°C to 150°C and then at 1°C/min to 200°C. The temperature was held constant for 10 minutes before final elevation at 5°C/min to 230°C for 5 minutes. The detector was set at 300°C.

Nitrogen was used as the carrier gas (1 mL/min). Twenty-nine fatty acid methyl esters were identified based on the retention times of the laboratory standards.

The identification of FAME was confirmed by mass spectroscopy. A procedural blank and esterified laboratory reference material of cod liver (LRM 144 and LRM 147) and esterified laboratory reference material of Orange Roughy (LRM 145) were analyzed with each batch of samples. A Restek FAME Standard or Nucheck Standard and EO23 were analyzed with each batch of samples to confirm chromatogram profiles and

component retention times. Laboratory reference materials were run with the samples and appropriate quality assurance checks made using Shewart Charts. The normalized area percentage was calculated on the basis of the twenty nine fatty acids.

Peaks were identified by comparison with the reference chromatograms. There should be no peak tailing or fronting and peaks were resolved, where possible.

To calculate normalized area in percentage, the data was collected and processed using a Perkin-Elmer Turbochrom Navigator, where peak areas were generated by Totalchrom software. Calculation results were based on conversion of peak area data to normalized area percentages. The Normalized area percent was determined for each component using the formula:

$$Normalized\ Area\ \% = \frac{Area[\mu V.s] for\ single\ identified\ component}{Sum\ total\ Area\ [\mu V.s]\ for\ all\ identified\ components} \times 100$$

Peak retention times of the various methyl esters were updated manually by comparison with the retention times in the reference materials (EO23, Nucheck, Restek and laboratory reference materials (LRMs)) analyzed with each batch of samples. Visual checks of peak integrations were made for each sample. LRMs were esterified and analyzed with each batch of samples as a check on the esterification procedure. Fatty acid methyl esters studied are shown in Table 1.1.1.

1.1.1.6 Confirmation of FAME identification by GC-MS

GC-MS using electron impact ionization confirmed FAME identified with the GC-FID. The GC-MS was a HP 6890 gas chromatograph interfaced with an HP 5793 selective detector and on-column injector. The column temperature was held at the initial oven temperature of 65°C for 2 minutes, then ramped at 20°C per minute to 150°C, then ramped at 1°C per minute up to 200°C and held for 10 minutes, then ramped at 10°C per minute to 220°C and finally held at 220°C for 10 minutes. Helium was used as the carrier gas at 0.7 mL min-1. The interface and MS source were set at 230°C and MS Quad was set at 150°C. The column used was a fused silica capillary column (0.25mm

i.d. x 30m length) coated with the polar DB-23 phase (film thickness, 0.25 µm, Agilent, UK).

1.1.1.7 Material Quality Control

Gloves were worn throughout the analytical procedure to prevent contamination from lipids present on the skin. Prior to use, analytical glassware was rinsed with *iso*-hexane which evaporated before procedure and rotary evaporator glassware was rinsed with chloroform.

All methods were validated by the replicate analysis of the standards and samples and with the analysis of laboratory reference materials, see (Méndez-Fernandez et al. 2014).

With each batch of samples, cod liver oil LRM and an Orange Roughy oil LRM (10mg) were analyzed. Cod liver oil was esterified to FAME and glycerol. Orange Roughy was esterified to FAME and fatty alcohols. Procedural blanks with BHT were analyzed with each batch of samples and neither fatty acids or fatty alcohols should be present in the procedural blanks. If any were detected, the batch was repeated.

Lipid samples were stored frozen and out of direct sunlight to limit lipid oxidation. Tissue samples selected for lipid extraction were stored frozen and allowed to thaw on the bench out of direct sunlight to avoid lipid oxidation.

The balances performance check was carried out daily. A new scalpel blade was used for each sample to prevent cross-contamination. All tools were cleaned with *iso*-hexane between samples and chloroform, if necessary.

1.1.1.7.1 Peak identification, quantitation and quality control

Every batch started with a couple of samples of *iso*-hexane and the standards EO23, NuCheck and Restek to confirm the GC retention times and for quality assurance.

EO23 fish oil, a long-standing reference material, developed to assess laboratory competency to determine fatty acid composition of fish oil, is used for several years to confirm FAME retention times.

EO23 was analyzed for FAME along with each batch, every 6-10 samples and used as a check on the retention times. The custom made standard NuCheck from NuCheck (Mn, USA) contain both FAME and fatty Alcohols and Restek Marine Oil contain FAME.

Laboratory reference materials, cod liver oil and Orange Roughy oil, were esterified and analyzed with each batch of samples as a check on esterification procedure, every 15 samples.

The data on the fatty acids composition obtained from the reference materials were transferred onto NWA Quality Analyst and control charts were produced for warning and action limits on GC-FID measurements.

1.1.1.7.2 Shewart charts - quality control

Shewart charts onto NWA Quality Analyst tested for quality control of comparability of the data. These charts check for stability of the analytical process where repeated measurements of a single quality characteristic are plotted against the sample number or time. Here, the normalized area percentage of selected fatty acids (14:0, 16:0, 16:1(n-7), 18:1(n-9), 20:5(n-3), 22:6(n-3)) derived from the repeated analysis of EO23 and LRMs is used for quality control of measurements.

The graph is described by a centre line that represents the mean value of the specific fatty acid from all samples of the standard. The upper and lower lines are the mean

plus and minus three standard deviations of the represented mean, which represents the Warning limit.

The lines above and below the mean are the mean plus and minus two standard deviations, respectively. These lines represent the Control limit, which are the range of variation expected in statistical control, i.e. consistent measurements. Control and warning limits are not fixed values. When many data points are added to a chart (more than 30 points) or if the analytical procedure changes, a better estimate of the variability of the sample set requires standard deviation adjustment.

Chart sample points represent a summary statistic (i.e. not single values) giving the mean for a sample of measurements of the quality characteristic, e.g. mean of each fatty acid by batch analyzed. The variation within these subgroups is minimized and variation between subgroups is maximized so the chart is more sensitive to shifts in the actual analytical process.

Values must have a random distribution around the centre line below warning and control limits (Figure 1.1.2, Figure 1.1.3, Figure 1.1.4). A point outside these controls limits or a systematic distribution in the measurements indicates a special cause of variation, i.e. a statistically unusual pattern of points implies out of control conditions. The method is out of control when two successive values exceed the warning limit, one value exceeds the control limit or 7 values in a row are on either side of the mean (centre line). With any of these conditions, the method was considered unstable so measurements were repeated.

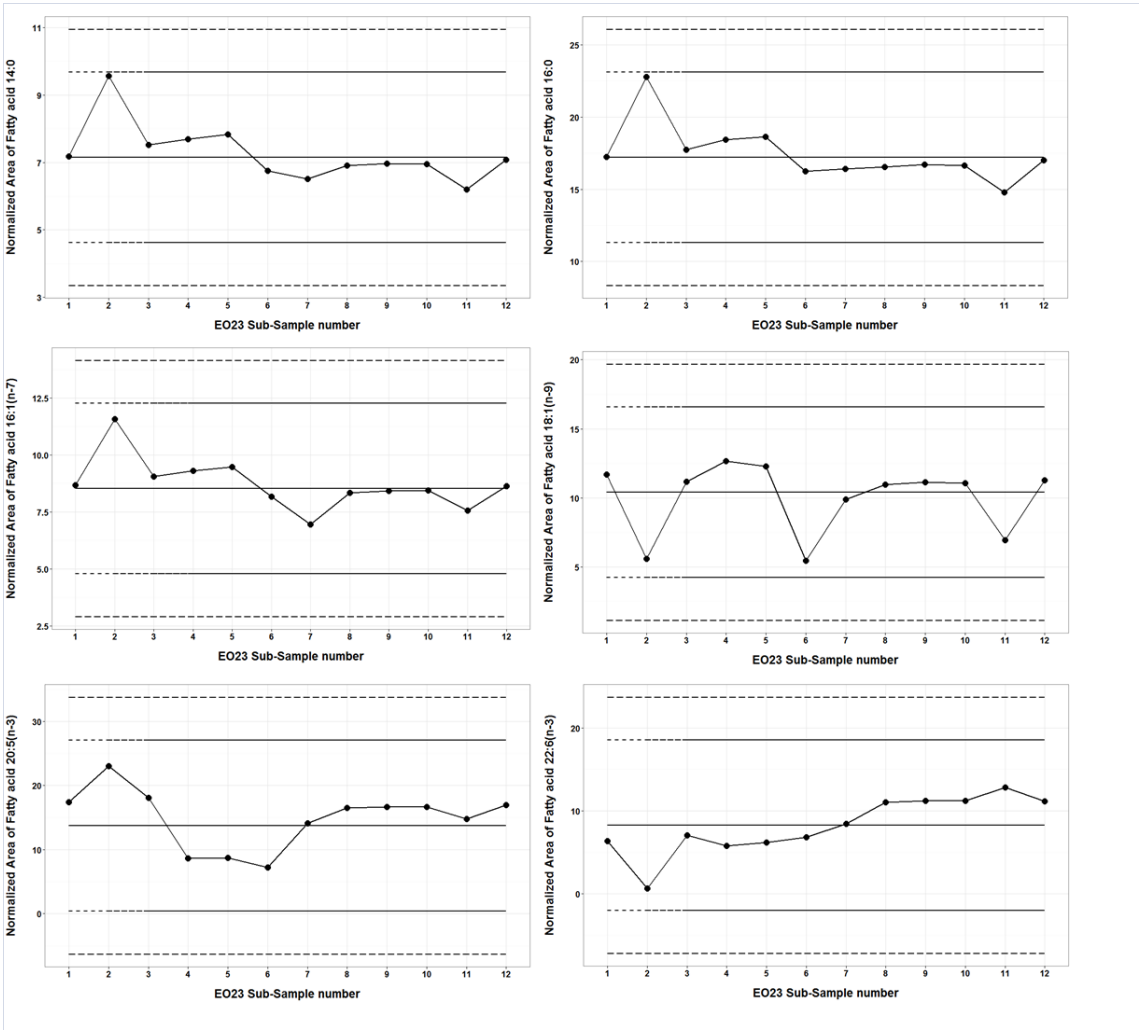

Figure 1.1.2 Shewart chart of fatty acids 14:0, 16:0, 16:1(n-7), 18:1(n-9), 20:5(n-3) and 22:6(n-3) for GC5890 of the standard EO23. The centre line represents the mean of the overall samples analyzed. Each point is a subgroup mean for each batch. The upper and lower dotted lines in the graph are the control limits (mean ± 3 standard deviations) and the upper and lower lines above and below the centre line are the warning limits (mean ± 2 standard deviations).

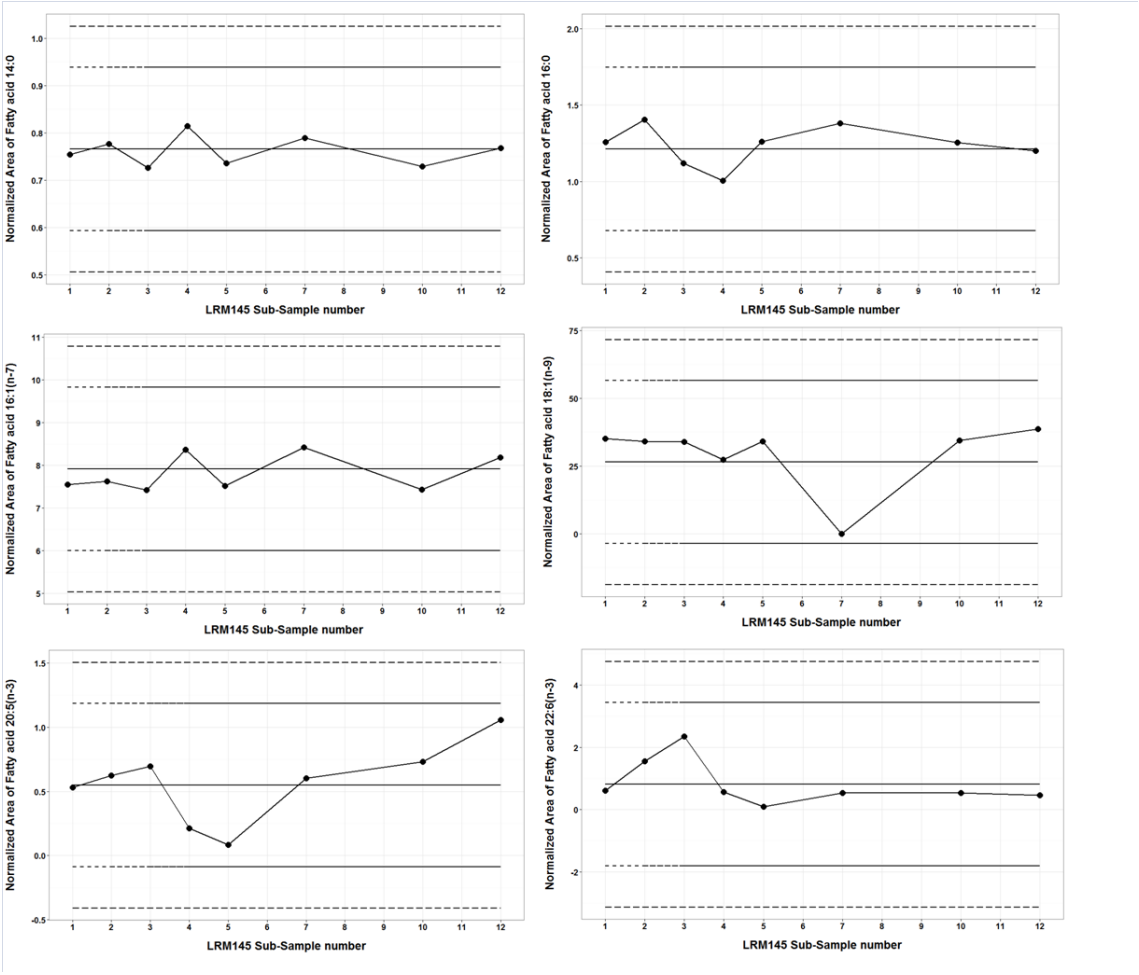

Figure1.1.3 Shewart chart of fatty acids 14:0, 16:0, 16:1(n-7), 18:1(n-9), 20:5(n-3) and 22:6(n-3) for GC5890 of the Laboratory standard Orange Roughy (LRM145). The centre line represents the mean of the overall samples analyzed. Each point is a subgroup mean for each batch. The upper and lower dotted lines in the graph are the control limits (mean ± 3 standard deviations) and the upper and lower lines above and below the centre line are the warning limits (mean ± 2 standard deviations).

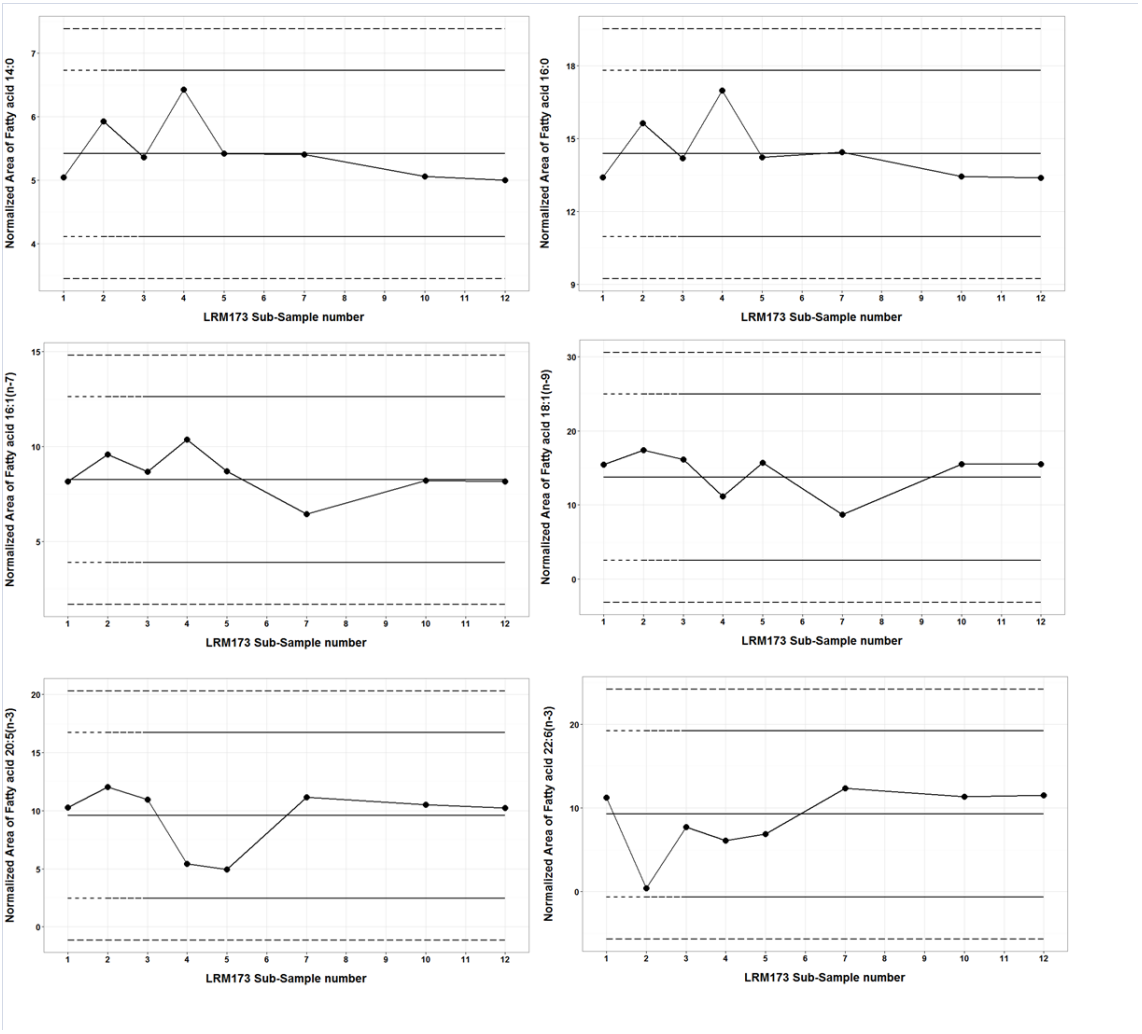

Figure1.1.4 Shewart chart of fatty acids 14:0, 16:0, 16:1(n-7), 18:1(n-9), 20:5(n-3) and 22:6(n-3) for GC5890 of the Laboratory standard cod liver oil (LRM173). The centre line represents the mean of the overall samples analyzed. Each point is a subgroup mean for each batch. The upper and lower dotted lines in the graph are the control limits (mean ± 3 standard deviations) and the upper and lower lines above and below the centre line are the warning limits (mean ± 2 standard deviations).~

1.1.2 Stable Isotopes

1.1.2.1 Introduction

Stable Isotope Analysis is a common technique to study trophic interactions, trophic positions, sources of energy and spatial and temporal changes in the ecosystem structure. These changes from anthropogenic or natural origin are of high importance to detect before any consequences for the ecosystem functioning and ultimately for ecosystem productivity (Hobson 1999, Pinnegar and Polunin 1999, MacNeil et al. 2005, Estrada et al. 2005, El-Sabaawi et al. 2009).

Stable isotope composition is estimated directly from tissues and differences are driven by isotopic differences among food sources, due to photosynthetic pathways, consumer-resource discrimination, fractionation or properties of the consumer (e.g. diet, trophic position, tissue type, taxonomic group, feeding guild, lipid content, marine versus terrestrial food sources, habitat conditions) (MacNeil et al. 2005, Nyssen et al. 2005, Stowasser et al. 2006, Carlier et al. 2007, Kharlamenko et al. 2008, Deudero et al. 2009, Cherel et al. 2009b, Boecklen et al. 2011, Pernet et al. 2012b).

The variation in isotopic signatures of food sources in complex estuarine and marine ecosystems and the multiple food sources that contribute to fish species require a complementary tool to enhance the analysis of trophic interactions and diet changes, as fatty acid analysis (Alfaro 2006, Petursdottir et al. 2008).

1.1.2.2 Isotope Chemistry and Terminology

Isotopes are naturally existing atoms of the same element with different mass that behave in a similar chemical way. Stable isotopes do not decay radioactively and many elements of biological importance (e.g. C, H, N, O, S) have two or more stable (nonradioactive) isotopes of which the lightest is usually in greater abundance. Each organism has an isotopic composition of the rare, heavy isotope (e.g. ^{13}C, ^{15}N) and the common, lighter isotope (e.g. ^{12}C, ^{14}N).

The proportion of stable isotopes relative to elemental atoms is measured in comparison to worldwide standards with a mass spectrometer and expressed as:

$$\delta R = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000$$

where R is the ratio of the heavy to the light stable isotope of the element, measured in parts per thousand (‰). δR is the heavy to light isotope ratio for the sample relative to an international standard. Delta units are per thousand (‰).

Smaller values are relatively "depleted" and higher values are relatively "enriched". Stable Isotope Standards (δ=0): PeeDee Belemnite (PDB) for isotopes ^{13}C and ^{12}C has a ratio of 0.0112372 ± 0.0000090; Atmospheric nitrogen for isotopes ^{15}N and ^{14}N has a ratio of 0.003663 ± 0.0000081 (De Lange and Van den Brink 2006).

Isotopes in this thesis are common in trophic ecology studies to elucidate on the flow of energy (e.g. primary producers, organic material) through the food web (Fry 2006, Boecklen et al. 2011):

- $\frac{^{13}C}{^{12}C}$ for primary production, trophic interactions, plant physiology;

- $\frac{^{15}N}{^{14}N}$ for trophic interactions and nutrients input.

1.1.2.3 Trophic level

Minagawa and Wada (1984) calculated an average fractionation at a single feeding process of 3.4‰, independent of habitat and trophic level. The bioaccumulation of δ^{15}N along a food chain occurs from the excretion of materials isotopically lighter than tissues and absorption of isotopically heavier materials (Del Rio et al. 2009). This value, though in constant debate, is still widely applied to estimate trophic level of consumers in many systems and corresponds to a one trophic level increment in δ^{15}N (Post 2002). The formula to calculate trophic position will be:

$$\Delta = 2 + (average\ \delta^{15}N_{consumer} - average\ \delta^{15}N_{primary\ producer})/3.4$$

The estimation of trophic position of consumers based on their δ^{15}N values misleads conclusions since different producers in the same area can differ on δ^{15}N values or δ^{13}C and δ^{15}N not be independent (Van der Zanden and Rasmussen 2001). The comparison of trophic position of organisms is only effective if the primary producers are the same or have similar δ^{13}C and δ^{15}N values. This way, to calculate a trophic position of an organism it is of great importance to provide a reliable baseline in space and time.

1.1.2.4 Isotopic discrimination or fractionation

Discrimination factor or "tissue to diet discrimination" (usually denoted by Δ) is the difference in stable isotope ratios between tissue of consumer and diet. When the diet changes in isotopic signature, the tissues of the consumer adjust and approaches a new equilibrium consistent with the new diet (Boecklen et al. 2011). The stable isotope ratio of the tissue results from chemical, physical and physiological processes. On the other hand, the term fractionation refers specifically to chemical reactions that cause differences in stable isotope ratios between an entire animal and its diet (Del Rio et al. 2009).

Discrimination factors vary among species, among tissues within a single species and among diets. Correct estimates of discrimination are a prerequisite to describe trophic interactions in a robust manner. The lack of diet experiments to estimate discrimination factors for each of the sources justifies the frequent use of the aforementioned $\delta^{15}N_{tissue\text{-}diet}$=3.4‰, which may differ considerably from the real values (Del Rio et al. 2009).

Tissues vary greatly in diet discrimination of $\delta^{13}C$ due to lipid content and amino acid composition. Lipid synthesis results in depleted $\delta^{13}C$, thus the recommendation for lipid extraction by several authors (DeNiro and Epstein 1977, Pinnegar and Polunin 1999, Stowasser et al. 2006).

1.1.2.5 Tissue turnover

Food assimilated by the cells reflects the isotopic signature of the food ingested and the time to change the isotopic signature of the animal tissue with a change of diet depends on the rates of growth and metabolism of tissues.

As turnover rate differs among tissues, it becomes possible to track a temporal change in diet. Tissues, such as liver, with high metabolic rate of protein turnover have an isotopic composition that reflect integration of recent dietary inputs; while muscle, with low metabolic rates of protein turnover have an isotopic composition that reflect integration of dietary inputs over longer time periods (Tieszen et al. 1983a, MacNeil et al. 2005, Estrada et al. 2005, Dalerum and Angerbjörn 2005, del Rio et al. 2009). Muscle of a whitefish was demonstrated to reflect diet isotopic composition with a 4-5 months lag time, while the liver reflected diet isotopic composition with a 1 month lag time (Perga and Gerdeaux 2005).

1.1.2.6 Carbon

Carbon isotope ratios are relative conservative from primary producer to apex predator, with an enrichment of 1‰ per trophic level. With these isotopes the diet and transfer of organic matter through a consumer food web becomes possible.

In terrestrial ecosystems, plants with C3 photosynthesis ($\delta^{13}C$ = -28‰) are clearly distinct from C4 ($\delta^{13}C$= -13‰) plants. $\delta^{13}C$ of aquatic plants or algae, depend on the source of dissolved inorganic carbon, the photosynthetic pathways and the supply of carbon (Fry 2006). Freshwater algae have values of $\delta^{13}C$= -20 to -45‰ and marine algae have values of $\delta^{13}C$= -19 to -24 ‰. For marine systems, $\delta^{13}C$ is depleted in pelagic sources and enriched in benthic sources (De Lange and Van den Brink 2006, Kharlamenko et al. 2008, Bouillon et al. 2011).

1.1.2.7 Nitrogen

The $\delta^{15}N$ values of primary producers in space and time are affected by nitrogen sources, nitrogen concentration and species succession of primary producers (especially nitrogen-fixers vs. non-nitrogen fixers) (Cabana and Rasmussen 1996) and the amino acids composition of primary producers. Tissues in the consumer differ greatly in $\delta^{15}N$ and hence in $\Delta^{15}N_{tissue\text{-}diet}$ with $\delta^{15}N$ amplified by the metabolic processes of consumers and trophic level (Del Rio et al. 2009).

Primary producers vary widely in $\delta^{15}N$ seasonally and between systems and to obtain baseline $\delta^{15}N$ values the best choice is the $\delta^{15}N$ values of long-lived primary consumers from the system under study (Cabana and Rasmussen 1996, Post 2002).

1.1.2.8 Stable Isotope Applications in Ecology

The stable isotope composition variability among and within tissues is useful to determine seasonal or preferential changes in food sources and track migrations (MacNeil et al. 2005).

The cause for variability is vital to understand the trophodynamics of the ecosystem and for the identification of anthropogenic or natural effects in aquatic systems that affect primary producers at the base of the food web (Pinnegar and Polunin 1999, Machás et al. 2003).

Estuaries are example of ecosystems under great anthropogenic pressures, e.g. dams (Morais 2008). These ecosystems at the interface of freshwater and marine environments support a great density and diversity of life (Sá et al. 2006, Connolly et al. 2009, Kostecki et al. 2010, Vinagre et al. 2011) and supply primary producers and terrestrial organic material for the base of coastal food webs.

Environmental variability including terrestrial input from pollution, sewage, stream modification (Machás et al. 2003, Morais 2008) has the most frequently negative outcome in the estuarine ecological biodiversity (Loneragan and Bunn 1999).

A great application for stable isotopes is the detection of spatial and temporal variability of the base of the food web on coastal habitats (Pernet et al. 2012b) that affect sustainability of biodiversity and productivity.

1.1.2.9 Stable Isotope Analysis

Materials and methods for the stable isotope analysis are described in each chapter.

1.1.3 Data treatment

Data was checked for normality (Shapiro-Wilk test) and homogeneity of variances (Bartlett test) prior to statistical tests. Fatty acids that contributed less than 5% to the fatty acid profile or without relevance as trophic markers were excluded from the analysis. No data transformation was applied to avoid giving artificial weight to minor fatty acids (Nyssen et al. 2005).

2 Diet shifts and importance of terrestrial organic material for fish food webs

2.1 Abstract

Commercial and recreational fisheries productivity in Guadiana estuary and adjacent coastal areas are affected by trophic interactions in fish trophic webs and, ultimately, by energy sources at the base of the food web. Stable isotopes and fatty acid trophic markers analyzed shifts in food web sources of energy for fish in the Guadiana estuary and offshore the Guadiana plume. Isotopic signatures of fish tissues, result from the combination of the food sources, varied greatly between the mid estuarine and the offshore environment. Enriched values of $\delta^{13}C$ (±19‰) in fish caught Offshore indicate marine phytoplankton and microphytobenthos as major energy sources at the base of the food web. Depleted $\delta^{13}C$ (± -22‰) in fish caught in Mid estuary indicate a possible mixture of primary producers, including estuarine microphytoplankton and microphytobenthos. Enriched $\delta^{15}N$ values in the Mid estuarine fish suggested a more prominent carnivory feeding behavior than for Offshore fish. Isotopic signatures highlighted differences in sources of nutrition for Mid estuary fish: ± -3‰ in $\delta^{13}C$ and ± +2 ‰ in $\delta^{15}N$. The analysis of Fatty Acids Trophic Markers (FATM) suggested a recent increase in carnivory. The combination of these techniques detected a short term diet shift in fishes within distinct environments and the current supply of terrestrial matter irrelevant as energy source of the studied fish. Isotopic differences in food sources strongly support spatial effects and dependency on local food sources for mid estuarine fish. Differences between tissues may result from the physiological processes of a constant diet from the same habitat. Conclusions on diet sources are only feasible if fishes were migration-restricted between habitats. Site-fidelity in mid estuary seems probable due to significant differences in isotopic values among habitats and among tissues within habitats.

2.2 Introduction

High fisheries productivity in estuarine ecosystems result from strong spatial and seasonal changes in environmental conditions (Pasquaud et al. 2008). Fisheries species in the Guadiana estuary are opportunistic predators. The wide variety of prey include benthic organisms, as amphipods, shrimps, polychaetes and small fish (Sá et al. 2006). This trophic flexibility reflects changes at lower trophic levels where human activities and natural variability impact the most and trigger trophic cascades with effects on higher consumers in this estuarine ecosystem (Käkelä et al. 2005).

Estuarine food sources are produced locally or driven by the water flow from other production sites (Connolly 2003b). Methods that study diet components include gut content analysis which details the latter ingested food items (Sá et al. 2006). Yet, this technique is unfeasible to describe temporal and spatial feeding behavior of opportunistic feeders (Pinnegar and Polunin 1999). Other techniques include stable isotope analysis (SIA) and fatty acids analysis (FAA).

Stable isotopes analysis estimate the origin of the ultimate source of primary production at the base of the food web that support fish growth ($\delta^{13}C$) (DeNiro and Epstein 1978, McCutchan et al. 2003, Connolly 2003b) and the relative trophic position of the fish in the trophic web ($\delta^{15}N$) (DeNiro and Epstein 1981, Minagawa and Wada 1984) and, seldom, the nitrogen source ($\delta^{15}N$) (McCutchan et al. 2003). $\delta^{13}C$ can indicate the habitat of a species, by discriminating carbon levels of different ecosystems, e.g. freshwater vs. marine, pelagic vs. benthic and offshore vs. inshore and latitudinal differences (Hobson 1999, Sierszen et al. 2003, Romanuk and Leavings 2005, Vinagre et al. 2011). The stable carbon and nitrogen isotope ratios in a consumer tissue are directly related to diet by a stepwise enrichment process per trophic level, about <1‰ in $\delta^{13}C$ (DeNiro and Epstein 1978) and 3‰ to 4‰ in $\delta^{15}N$ (Minagawa and Wada 1984). In fish, the enrichment per trophic level has an average of 2.79‰ for $\delta^{15}N$ (Sweeting et al. 2007b) and 1.5‰ for $\delta^{13}C$ (Sweeting et al. 2007a).

Pitfalls of stable isotope analysis are the ambiguity of $\delta^{13}C$ to trace fish food sources, if different dietary sources have similar values (Connolly 2003b) and the requisite of a baseline $\delta^{15}N$ value to estimate the fish trophic level (El-Sabaawi et al. 2009).

The analysis of fish fatty acids profile traces energy transfers in the trophic web, predator-prey relationships and even the feeding habitat (Dalsgaard et al. 2003, Petursdottir et al. 2008). Certain taxa of primary producers and zooplankters synthesize specific fatty acids that transfer through the food chain mostly unchangeable, e.g. gondoic acid (*cis*-11-eicosenoic acid, 20:1(n-9)) and cetoleic acid (*cis*-11-docosenoic acid, 22:1(n-11)) are only biosynthesized *de novo* by copepods. These specific fatty acids are designated as Fatty Acid Trophic Markers (FATM) (e.g. vascular plants: linoleic acid (*cis*-9,12-octadecadienoic acid; 18:2(n-6)); copepods: oleic acid (*cis*-9-octadecenoic acid; 18:1(n-9)), gondoic acid (20:1(n-9) and cetoleic acid (22:1(n-11); salt marsh and vascular plants: α-linolenic acid (*cis*-9,12,15-octadecatrienoic acid; 18:3(n-3)); diatoms: palmitoleic acid (*cis*-9-hexadecenoic acid; 16:1(n-7)) and EPA or timnodonic acid (*cis*-5,8,11,14,17-eicosapentaenoic acid; 20:5(n-3)); dinoflagellates: DHA (*cis*-4,7,10,13,16,19-docosa hexaenoic acid; 22:6(n-3)); green algae: asclepic acid (*cis*-11-octadecenoic acid; 18:1(n-7)). These trophic markers and some fatty acid ratios derive relative trophic positions and dietary quality: carnivory feeding behavior (arachidonic acid, ARA (*cis*-5,8,11,14-eicosatetraenoic; 20:4(n-6))) and 18:1(n-9)/18:1(n-7) and PUFA/SFA (El-Sabaawi et al. 2009); diet dominated by diatoms (bacillariophytes) as primary producers: ratio of palmitoleic acid, 16:1(n-7) and palmitic acid (hexadecanoic acid, 16:0), 16:1(n-7)/16:0>1 (Auel et al. 2002, Dalsgaard et al. 2003); food source derived from vascular plants: 18:2(n-6)+18:3(n-3) (Budge and Parrish 1998); copepods consumption: sum of gondoic acid (20:1(n-9)) with erucic acid (*cis*-13-docosenoic acid, 22:1(n-9)) and cetoleic acid (22:1(n-11)), 20:1(n-9)+22:1(n-9/11); dominance of diatoms over dinoflagellate as primary producers in the food web: EPA/DHA>1 and $\sum C_{16}/\sum C_{18}$ (Budge and Parrish 1998, Auel et al. 2002, Dalsgaard et al. 2003, Alfaro 2006, Kelly and Scheibling 2012).

Fatty acid analysis pitfalls are estimates derived from mixtures of food sources, as DHA from dinoflagellates and from feeding behavior of carnivory (Dalsgaard et al. 2003).

Time of year, sex, geographic location and specific tissue under investigation (MacNeil et al. 2005) influence fatty acid composition and FATM become less clear with increasing trophic levels (Auel et al. 2002).

A powerful tool to better demonstrate seasonal dynamics of fish food sources in the estuarine environment and if different quality food sources affect trophic interactions, is the combination of stable isotopes analysis and fatty acids analysis in different fish tissues (Tieszen et al. 1983a, Stowasser et al. 2006, Kakela et al. 2007, Petursdottir et al. 2008).

The specific tissue turnover rate is the change in tissue composition due to growth and metabolism (MacAvoy et al. 2001, Phillips and Eldridge 2005, Stowasser et al. 2006). Tissues with higher metabolic rate reflect more rapidly diet shifts, thus, latter dietary features (Tieszen et al. 1983a, MacNeil et al. 2005) with minor modification in fatty acids composition and stable isotopes composition between predator and prey (Stowasser et al. 2006). Since diet supplies the majority of stable isotopes and fatty acids to the body (Dalsgaard et al. 2003, MacNeil et al. 2005, Stowasser et al. 2006), the contrast between a high metabolic rate tissue, as liver tissue, and a lower metabolic rate tissue, as muscle tissue, will detect a short-term variability of the diet (MacNeil et al. 2005, Estrada et al. 2005, Perga and Gerdeaux 2005, Phillips and Eldridge 2005).

The importance to detect shifts in food sources that sustain fisheries species enables to establish fisheries management procedures and predict fisheries productivity in the region. In the coastal area adjacent to Guadiana estuary there are important commercial and recreational fisheries species. Landings in the commercial fisheries in the area had a net value up to 3.5 million Euros in 2014, the second largest in the Algarve region (www.ine.pt).

This study applied stable isotope analysis and fatty acids analysis for multiple-tissue comparison to assess short-term trophic dynamics in coastal and estuarine fish caught in an estuary. The main question is on the use of the estuary by coastal fish and habitat

trophic connectivity. Is the estuarine energetic supply to the coastal fish relevant enough to create habitat trophic connectivity for coastal fishes?

2.3 Materials and methods

2.3.1 Study site

The Guadiana river has an estuary in the Southeastern Portugal (37º11'N, 7º25'W), as shown in (Barbosa et al. 2010). The estuary is 70 km long, with the upper estuary at Mértola, i.e. river flow with tidal influence but salinity close to zero. The middle estuary, ca. 38 km upstream, is a transition zone with brackish water and Alcoutim is where seawater reaches its maximum upstream distance. The lower estuary has water with salinity similar to seawater and an adjacent salt marsh area (Chícharo et al. 2006, Sá et al. 2006, Barbosa et al. 2010).

Downstream fish habitats are affected by hydro-sedimentary processes that occur along the river, as well as upstream migration behavior and trophic dynamics, which in turn constrain estuarine and coastal fisheries production. Examples of these hydro-sedimentary processes are transport of sediments that affect the estuarine plume, which is a cue for upstream migration of coastal marine fish for spawning or nursery estuarine areas. The reduction of nutrients exported downstream to coastal and offshore environments are also affected by the transport of sediments (Morais 2008).

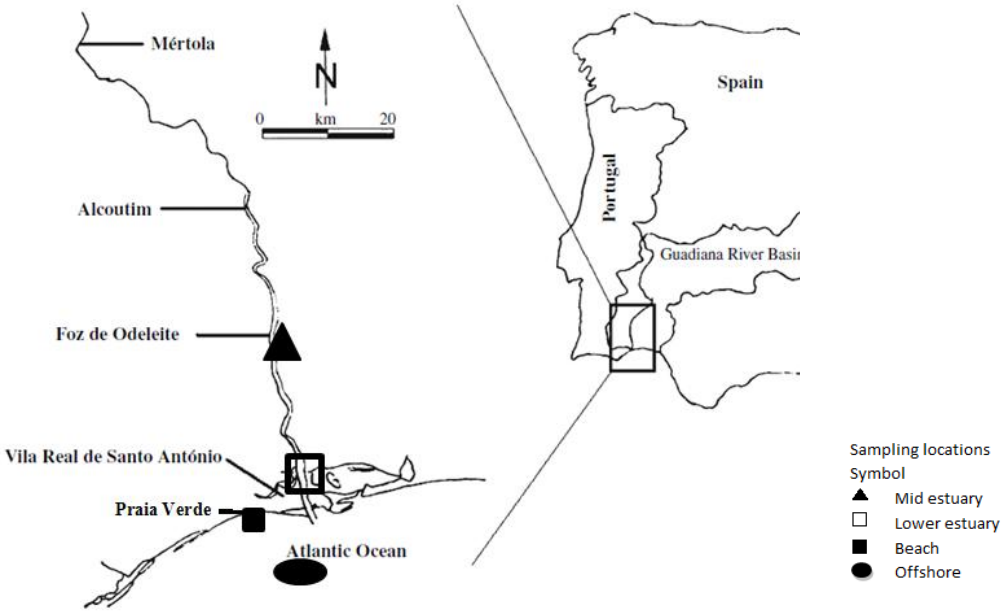

Figure 2.3.1 Map of fish sampling sites in the Guadiana estuary, offshore the river plume and in the coast (adapted from Domingues et al. 2011).

The Algarve has a temperate climate of Mediterranean influence and the Guadiana river has freshwater flow with strong seasonal and yearly variability, e.g. in the summer months is usual low river flow and higher salinities (Cravo et al. 2006, Morais 2008).

The water temperature varies between 10ºC in the winter and 28ºC in the summer. Rainfall is often inexistent in summer in contrast with pronounced winter rainfall events (Barbosa et al. 2010). The freshwater retention increased up to 81% due to a recent dam, 150 km upstream, and now pulse water discharges are more frequent in the winter and more reduced in the dry summer months (Morais et al. 2009).

2.3.2 Sample collection

2.3.2.1 Organic matter

Suspended particulate matter (SPM) and sediment particulate matter were collected in summer 2011, in the Guadiana estuary: Mid estuary (Odeleite) and Lower estuary (river mouth); and in a coastal beach (Praia Verde, 7 km west of the river mouth) (Figure 2.3.1).

Freshwater flow from the Guadiana River and oceanic water from the Atlantic Ocean affected the sites salinity differently. Mértola was only influenced by freshwater (salinity: 0.19±0.01), as the maximum upstream saltwater intrusion is Alcoutim, 40 km downstream; Odeleite was most influenced by freshwater (salinity: 0.21±0.02); the river mouth was intermediate between the two water masses (salinity: 22.14 ±4.64); and the coastal beach was outside the river freshwater outflow influence (salinity: 34.28±1.59).

Samples, three bottles of water and three sediment samples (top 1-2cm off the surface layer of the sediment), were taken at the same time of the day, in June for SPM and sediment particulate matter and in September for SPM.

At the laboratory, each water sample was pre-filtered through a 200µm mesh to remove zooplankton and detritus, and then filtered on pre-combusted (500^{o}C for 4h) GF/C glass microfiber filter under moderate vacuum. The sediment was mixed in filtered water from the same location and sieved through a 200µm to remove detritus and larger particles, then filtered on a pre-combusted (500^{o}C for 4h) GF/C glass microfiber filter under moderate vacuum. Filters were frozen at -20^{o}C until analysis.

2.3.2.2 Fish samples

Fish collected from three areas (Figure 2.3.1): Mid-estuary of the Guadiana River; Lower estuary of the Guadiana River and Offshore (12-15 nm from the Guadiana River plume) between autumn 2010 and summer 2011 were captured with different gears: long lines, gillnets and hooks.

At the laboratory, muscle fish tissue and liver fish tissue were extracted. Muscle tissue fatty acid composition and stable isotopic composition reflect former diet over a longer period of time, compared to liver tissue which reflects latter feeding (Stowasser et al. 2006).

Samples were pooled for individuals of same species and sizes, captured at the same time and location. Pooled samples were composed of tissues of two or three individuals and comprised only individuals of same size to avoid ontogenic changes in diets. Individuals of different species were analyzed separately. Samples were stored frozen at -20^{o}C prior to biochemical analyses.

2.3.3 Sample analyses

2.3.3.1 Fatty acids analyses

Fatty acid composition in fish was determined for 24 samples of fish tissues, of which n=15 were pooled samples. Lipids were isolated from fish tissues with a modification of the method of (Folch et al. 1957) and using a chloroform-methanol solvent (2:1 v/v) mixture with BHT (butyl hydroxyl toluene).

The organic layer obtained by centrifugation was evaporated and resulting lipids were converted into fatty acid methyl esters (FAMEs) by transesterification: lipid extract was transferred to vials where distilled toluene (1mL) and sulphuric acid in methanol (1% v/v) were added; the vials were shaken and incubated at 50°C overnight in a heating block. Aqueous sodium chloride (5% w/v) was added and the methyl esters were extracted into 2x *iso*-hexane (5mL). The combined organic layers were washed with 2% w/v potassium bicarbonate (4mL) and dried over anhydrous sodium sulphate at -20°C until analysis. The extracts were diluted to a concentration of 1mg/mL prior to analyses.

The FAME in the samples were analyzed by gas chromatography with flame ionization detection (GC-FID) on an Agilent (Hewlett-Packard) 5890 Series II gas chromatograph, fitted with a fused silica capillary column (0.25mm i.d. x 30m) coated with a 0.25µm film of 50% cyanopropyl. Injections of 1µL of the methyl esters dilution were made using a cool, on-column auto injector at 60°C.

The oven temperature was ramped at 25°C/min to 150°C and then 1°C/min to 200°C, and held for 10 minutes before final elevation at 50°C/min to 230°C and held for 5 minutes. The detector was set at 300°C. Nitrogen was the carrier gas (1mL/min) (Webster et al. 2006).

29 fatty acid methyl esters were identified based on the GC retention times of the laboratory standards. Each batch of samples included procedural blanks and laboratory reference material.

Assurance checks were made with Shewart Charts from the laboratory reference material data and warning and action limits highlighted analytical procedures.

The data are expressed as normalized area percentage, i.e. percentages of the total area of the 29 fatty acid methyl esters. The fatty acids were further classified according to number of double bonds between the individual carbon atoms of the fatty acid chain: no double bonds: saturated fatty acids (SFA); one double bond: monosaturated fatty acid (MUFA); more than one double bond: polyunsaturated fatty acid (PUFA).

The input of organic sources to the fish food webs in the fishing areas was deduced from the relative abundance of typical Fatty Acid Trophic Markers (FATM) in fish tissues.

2.3.3.2 Stable isotopes analyses

Stable isotopes were analyzed for liver and muscle tissue, of 48 fish samples. Tissue samples, delipidified from the fatty acids analysis, were kept at -20°C until further analyses.

Delipidified samples were preferred since lipids are isotopically lighter than proteins and lipid content results in $\delta^{13}C$ decrease (DeNiro and Epstein 1977, Tieszen et al. 1983a, Nyssen et al. 2005).

Thawed samples were dried at 60°C for a minimum of 24h until constant weight. Grinding and homogenization of samples was done with a mortar and pestle to a fine powder.

A weight of 0.6mg±10% of each sample powder was placed into pre-weighed 6x4 mm SerCon tin capsules, which were folded and compacted and kept in a labeled tray in a dessicator until isotopic analysis.

SPM and sediment particulate matter provided a baseline of stable isotope signatures of the estuarine ecosystem.

The filters dried at 60°C for 48h, were not acidified, as preliminary analysis indicated absence of carbonates that could affect $\delta^{13}C$ values.

In a SerCon Carbon and Nitrogen Isotope Analyser coupled with an element analyser (IRMS, isotope ratio mass spectrometer) samples were combusted for isotopic composition measurement.

Carbon and nitrogen stable isotope ratios were analyzed with the resulting $CO_2(g)$ and $N_2(g)$.

Duplicate samples were run at random and internal laboratory standards ran at regular intervals for each batch.

$\delta^{13}C$ and $\delta^{15}N$ are reported as per mil (‰) difference of isotopic ratios of the sample from isotopic ratios of international standards reference materials (Vienna Pee Dee Belemnite for carbon and atmospheric N_2 for nitrogen). $\delta^{13}C$ and $\delta^{15}N$ were estimated with the formula:

$$\delta R = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000$$

Where $R=^{13}C/^{12}C$ or $R=^{15}N/^{14}N$, i.e. the ratio of heavy to light isotope in the sample. Values of isotopic composition were raw mass spectrometry δ estimates relative to laboratory working standards, which were adjusted to international standards. USGS40 was the reference material for stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) of unknown carbon and nitrogen-bearing substances (Qi et al. 2003).

$\delta^{13}C$ values were relative to VPDB (Vienna PeeDee Belemnite) (Coplen et al. 2006) with a precision of <0.2‰. $\delta^{15}N$ values were relative to atmospheric nitrogen, which is isotopically homogeneous (Mariotti 1983) with a precision of <0.4‰.

Trophic position (Δ) was expressed as $\Delta=(\delta^{15}N_{tissue}- \delta^{15}N_{SPM})/3.4+2$, where Δ was a continuous measure of trophic position, $\delta^{15}N_{tissue}$ was the $\delta^{15}N$ of the fish tissue, $\delta^{15}N_{SPM}$ was the $\delta^{15}N$ of SPM averaged over all samples and 3.4 is the degree of nitrogen fractionation (‰) observed for each trophic level according to (Minagawa and Wada 1984) and (Post 2002).

2.3.4 Statistical analysis

Data based on the identified 29 fatty acids, FATM and stable isotopic composition was analyzed with the program R (packages: "plyr", "reshape2" and "ggplot2") (R Core Team 2013).

Minor fatty acids, i.e. less than 5% contribution to the fatty acid profile and without relevance as trophic markers, were excluded. No data transformation was applied as fatty acids that contribute a small percentage to the total composition did not feature heavily in the diet. Giving artificial weight to these minor fatty acids by applying a transformation would therefore be inappropriate (Nyssen et al. 2005).

FATM and stable isotopes means and SDs were plotted per tissue and area.

To determine differences between tissues pairwise t-tests were performed between fish liver and muscle tissues for fatty acids, FATM and stable isotopes.

Normality of the data was checked by Shapiro-Wilk test.

Two-factor ANOVA, followed by Tukey-Kramer post hoc analysis assessed the interaction effect of tissue and fishing area, on each fatty acid, FATM and stable isotopes. Non-paired t-test tested differences within tissues and among fishing areas.

Multivariate analysis of variance (MANOVA) tested differences in overall fatty acid composition among fish due to interaction of tissue and fishing area by examining whether mean differences among groups occurred by chance. MANOVA assumed the data were multivariate normal and the covariance matrices were homogeneous, e.g. (Kelly and Scheibling 2012).

$\delta^{13}C$ and $\delta^{15}N$ were plotted for each species and respective fishing area.

Spearman product-moment correlation was calculated for fatty acids, FATM and stable isotopes between fish muscle and fish liver, and between each tissue and $\delta^{15}N$, $\delta^{13}C$ and Δ.

2.4 Results

2.4.1 Sample composition

Fifteen species composed the catches in the Guadiana estuary and adjacent coastal waters, off Guadiana river plume, with a total of 24 samples, individual or pooled (Table 2.4.1).

The species captured were *Argyrosomus regius* (Asso, 1801), *Chelidonichthys lucernus* (Linnaeus, 1758), *Chelidonichthys obscurus* (Walbaum, 1792), *Citharus linguatula* (Linnaeus, 1758), *Dentex* sp., *Dicentrarchus labrax* (Linnaeus, 1758), *Dicentrarchus punctatus* (Bloch, 1792), *Dicologlossa cuneata* (Moreau, 1881), *Diplodus annularis* (Linnaeus, 1758), *Halobatrachus didactylus* (Bloch & Schneider, 1801), *Scomber japonicus* Houttuyn, 1782, *Sparus aurata* Linnaeus, 1758, *Spondyliossoma cantharus* (Linnaeus, 1758), *Trachinotus ovatus* (Linnaeus, 1758), *Trachinus draco* Linnaeus, 1758.

Mostly benthic carnivores, these species feed on bivalves, crustaceans and other fish. The exceptions were *Scomber japonicus*, a pelagic feeder and *Sparus aurata*, a benthic omnivore (Froese and Pauly 2008). These species have generally a great interest in the local economy and are commercially captured in coastal areas.

Table 2.4.1 Summary of fish samples analyzed. The letter X represented one sample, with a single individual or a pool of individuals with the same size (n=2 or n=3) for each species, per year and estuary sampling location.

	Guadiana River estuary									
	Mid estuary				Lower estuary				Offshore	
	2010		2011		2010		2011		2011	
Species	single sample	pooled sample	single sample	pooled sample	single sample	pooled sample	single sample	pooled sample	single sample	pooled sample
Argyrosomus regius	x									
Chelidonichthys lucernus										x
Chelidonichthys obscurus									x	
Citharus linguatula										x
Dentex sp.										x
Dicentrarchus labrax		xxx			x					
Dicentrarchus punctatus		xx					x			
Dicologlossa cuneata									x	
Diplodus annularis										x
Halobatrachus didactylus							x			x
Scomber japonicus								x		
Sparus aurata		x			x		x			
Spondyliossoma cantharus									x	
Trachinotus ovatus								x		
Trachinus draco								x		x

2.4.2 Fatty Acid composition in fish liver and fish muscle

The fatty acid composition of fish liver and fish muscle, shown in Table 2.4.2, was higher in concentrations of palmitic acid (hexadecanoic acid; 16:0), oleic acid (18:1(n-9)) and DHA (22:6(n-3)). Other relevant fatty acids were palmitoleic acid (16:1(n-7)), stearic acid (octadecanoic acid (18:0)), asclepic acid (18:1(n-7)), ARA (20:4(n-6)) and EPA (20:5(n-3)).

Table 2.4.2 FAME components ±1SD in fish liver and fish muscle for overall samples. Data were expressed as normalized percentages (those without uncertainty calculated are asterisked).

Fatty Acids and Fatty Acid Trophic Markers	Liver n=24		Muscle n=24	
	Mean	SD	Mean	SD
14:0	2.50 ±	1.77	1.23 ±	0.80
14:1(n-5)	0.15 ±	0.15	0.14 ±	0.13
15:0	0.58 ±	0.22	0.45 ±	0.10
16:0	23.34 ±	3.42	20.00 ±	2.63
16:1(n-7)	6.11 ±	3.74	2.99 ±	2.08
16:2*	0.27 ±	0.27	0.50 ±	1.40
16:3*	0.09 ±	0.16	0.10 ±	0.18
16:4*	0.05 ±	0.07	0.12 ±	0.16
17:0	1.03 ±	0.64	0.79 ±	0.20
18:0	7.38 ±	2.19	7.21 ±	1.55
18:1(n-9)	16.86 ±	10.39	9.89 ±	4.46
18:1(n-7)	6.60 ±	8.08	3.21 ±	3.38
18:2(n-6)	0.88 ±	0.46	0.93 ±	0.43
18:3(n-6)	0.20 ±	0.10	0.11 ±	0.05
18:3(n-3)	0.48 ±	0.25	0.36 ±	0.20
18:4(n-3)	0.15 ±	0.16	0.12 ±	0.16
20:0	0.30 ±	0.17	0.28 ±	0.20
20:1(n-11)	0.90 ±	0.99	0.70 ±	0.31
20:1(n-9)	0.83 ±	0.59	0.38 ±	0.20
20:2(n-6)	0.41 ±	0.21	0.35 ±	0.20
20:3(n-3)	0.22 ±	0.15	0.18 ±	0.10
20:4(n-6)	3.57 ±	2.05	4.84 ±	2.20
20:4(n-3)	0.38 ±	0.14	0.40 ±	0.17
20:5(n-3)	5.53 ±	2.00	9.10 ±	2.75
22:1(n-9/11)	0.29 ±	0.50	0.39 ±	0.69
21:5(n-3)	0.08 ±	0.11	0.08 ±	0.11
22:5(n-3)	3.00 ±	2.31	3.86 ±	1.93
22:6(n-3)	16.68 ±	9.69	28.37 ±	7.56
24:1(n-9)	1.13 ±	0.92	1.24 ±	1.27
SFA	35.16 ±	4.47	30.68 ±	4.14
MUFA	32.84 ±	12.67	19.90 ±	6.66
PUFA	31.73 ±	13.82	48.91 ±	9.09
PUFA/SFA	0.93 ±	0.45	1.64 ±	0.44
16:1(n-7)/16:0	0.25 ±	0.14	0.15 ±	0.12
18:1(n-9)/18:1(n-7)	4.05 ±	2.81	4.42 ±	2.92
18:2(n-6)+18:3(n-3)	1.36 ±	0.65	1.30 ±	0.60
20:1(n-9)+22:1(n-9/11)	6.20 ±	6.00	2.45 ±	2.01
20:5(n-3)/22:6(n-3)	0.40 ±	0.20	0.34 ±	0.13
22:6(n-3)/20:5(n-3)	3.00 ±	1.57	3.38 ±	1.33
∑C16	29.85 ±	6.01	23.72 ±	4.18
∑C16/∑C18	0.96 ±	0.23	1.13 ±	0.25
∑C18	32.54 ±	9.08	21.84 ±	4.82

Fish liver was significantly richer in 16:0, 16:1(n-7), 18:1(n-9) ($p<0.001$), 18:1(n-7) ($p<0.01$), SFA ($p<0.01$) and MUFA ($p<0.001$). Fish muscle was significantly richer in 20:5(n-3) and 22:6(n-3) ($p<0.001$), 20:4(n-6) ($p<0.01$) and PUFA ($p<0.001$). Fatty acids markers of terrestrial matter incorporated in the food web, as linoleic acid (18:2(n-6)) and α-linolenic acid (18:3(n-3)), had negligible concentrations in both fish tissues (< 1%).

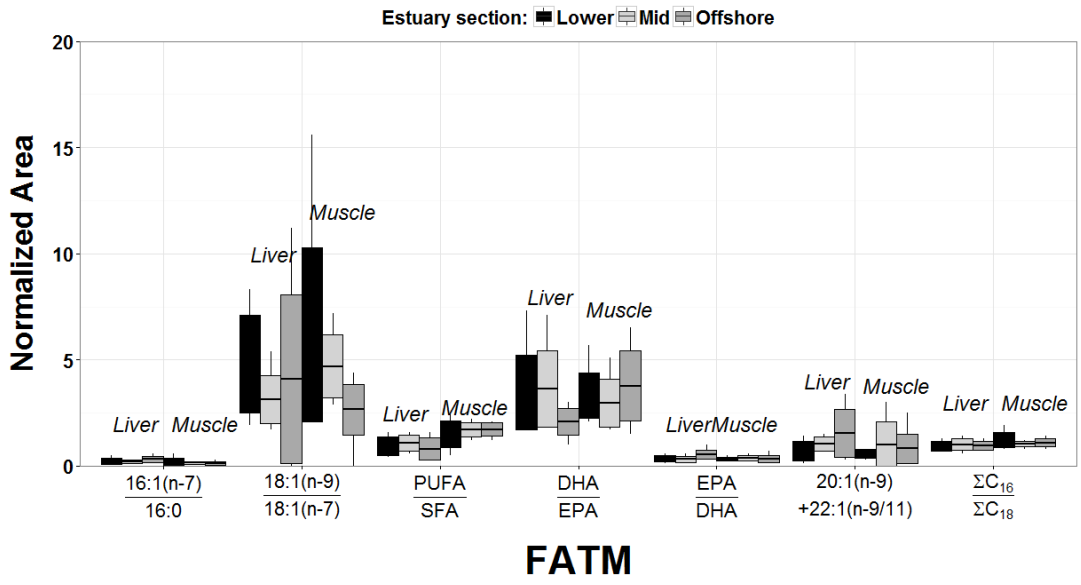

Figure 2.4.1 Intraspecific differences in Fatty Acid Trophic Markers (FATM) ratios of dietary quality in fish from the Guadiana lower and mid estuary and Offshore. Boxplots represent the mean ± 1SD and whiskers represent the max and min values.

Both tissues had consistent FATM typical of carnivory along the estuarine gradient and Offshore, e.g. 22:6(n-3)/20:5(n-3) or DHA/EPA; 18:1(n-9)/18:1(n-7); PUFA/SFA; 20:4(n-6) (Figure 2.4.2 and Figure 2.4.2).

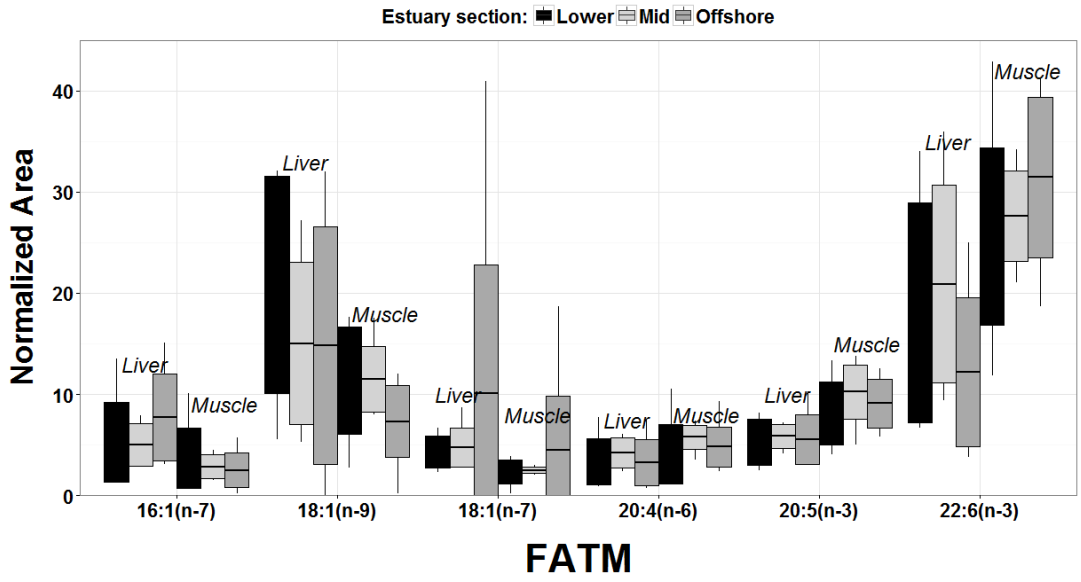

Figure 2.4.2 Intraspecific differences in Fatty Acid Trophic Markers (FATM) of dietary quality in fish from the Guadiana lower and mid estuary and Offshore. Boxplots represent the mean ± 1SD and whiskers represent the max and min values.

Copepod-based diet indicators, 18:1(n-9), higher in fish liver ($p<0.001$) and dinoflagellate-based diet indicators, 22:6(n-3), higher in fish muscle ($p<0.001$), suggested a diet richer in dinoflagellates and copepods as the main energy source.

Diatom trophic markers varied significantly in opposite proportions in the liver tissue: 20:5(n-3) decreased ($p<0.001$) and 16:1(n-7) increased ($p<0.001$).

Green algae trophic marker, 18:1(n-7), was present in both fish tissues, with higher proportions in the liver tissue ($p<0.01$) and more evident for Offshore fish (Figure 2.4.2).

FATM ratios as 16:1(n-7)/16:0 were significantly higher in fish liver ($p<0.01$), PUFA/SFA ($p<0.001$) and $\sum C_{16}/\sum C_{18}$ ($p<0.01$) were significantly higher in fish muscle, and 18:1(n-9)/19:1(n-7), 20:1(n-9)+22:1(n-9/11), EPA/DHA and DHA/EPA showed no significant differences between tissues ($p>0.05$).

The MANOVA showed overall significantly different fatty acid composition between tissues ($p<0.001$) but no effect of fishing area ($p>0.05$) nor interaction of fishing area or tissue ($p>0.1$) on fatty acid composition.

2.4.3 Stable isotope composition analysis in fish liver and fish muscle

Mid estuary and Offshore samples were compared and Lower estuary was excluded as it was represented by a single sample.

For Mid estuary fish liver, $\delta^{15}N$ mean was 14.16‰ ± 2.6‰ while Offshore fish liver $\delta^{15}N$ mean was 12.7‰ ± 1.7‰. For Mid estuary fish muscle, $\delta^{15}N$ mean was 16.74‰ ± 2.1‰ while Offshore fish muscle $\delta^{15}N$ mean was 13.35‰ ± 1.2‰ (Figure 2.4.3).

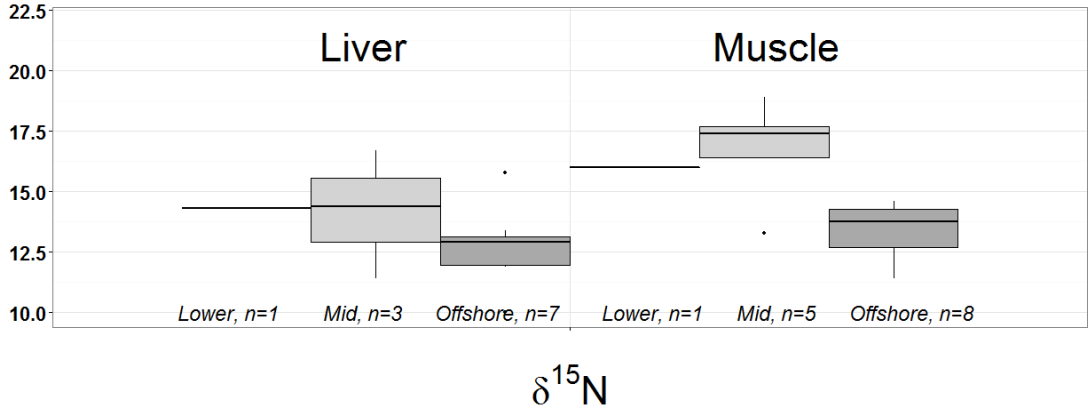

Figure 2.4.3 Intraspecific differences in $\delta^{15}N$ of dietary quality in fish from the Guadiana Lower and Mid estuary and Offshore represented by boxplots.

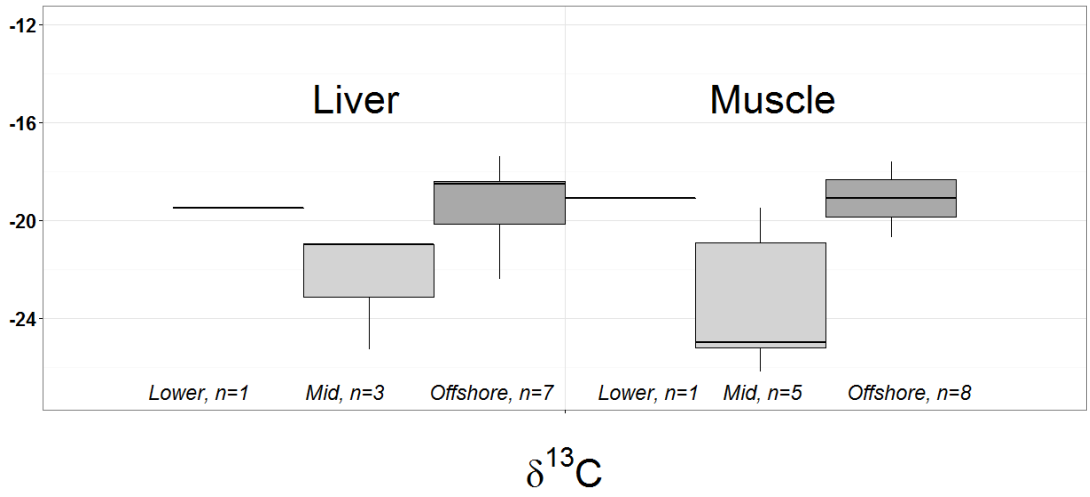

Figure 2.4.4 Intraspecific differences in $\delta^{13}C$ of dietary quality in fish from the Guadiana Lower and Mid estuary and Offshore represented by boxplots.

Between areas, for Mid estuary fish liver, $\delta^{13}C$ mean was -22.43‰± 2.48‰ while Offshore fish liver, $\delta^{13}C$ mean was -19.34‰±1.68‰.

For Mid estuary fish muscle, $\delta^{13}C$ mean was -23.36‰±2.96‰ while Offshore fish muscle, $\delta^{13}C$ mean was -19.15‰± 1.09‰

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Variability in $\delta^{13}C$ values, either in fish muscle and fish liver, were specifically higher in Mid estuary samples, as shown in Figure 2.4.4.

Pairwise t-tests indicated fish muscle was significantly richer in $\delta^{15}N$ ($p<0.001$) than fish liver. $\delta^{13}C$ values were not significantly different between tissues ($p>0.5$).

$\delta^{15}N$ in fish muscle was lower Offshore than in Mid estuary ($p<0.05$) but $\delta^{15}N$ in fish liver among areas were not statistically different ($p>0.1$). $\delta^{13}C$ in fish muscle was higher Offshore than in Mid estuary ($p<0.05$) but $\delta^{13}C$ in fish liver among areas were not statistically significant ($p>0.01$).

The range of values of $\delta^{15}N$ and $\delta^{13}C$ of fish liver and fish muscle were distinct among fishing areas and $\delta^{15}N$ and $\delta^{13}C$ values were significantly different for fish muscle

(p<0.05) within fishing area. The different tissues showed very similar ranges for the isotopic ratios, e.g. wide range for Mid estuary and narrow range for Offshore.

Fish trophic levels between 2.1 and 3.5 derived from estuarine Suspended Particulate Matter (SPM) δ^{15}N mean value of 8.80‰±1.69‰ of samples from mid and lower estuary. These low values of trophic levels were excluded for further analysis due to a very likely influence of anthropogenic effects (Machás et al. 2003). SPM δ^{13}C mean value was -32‰±6.77‰.

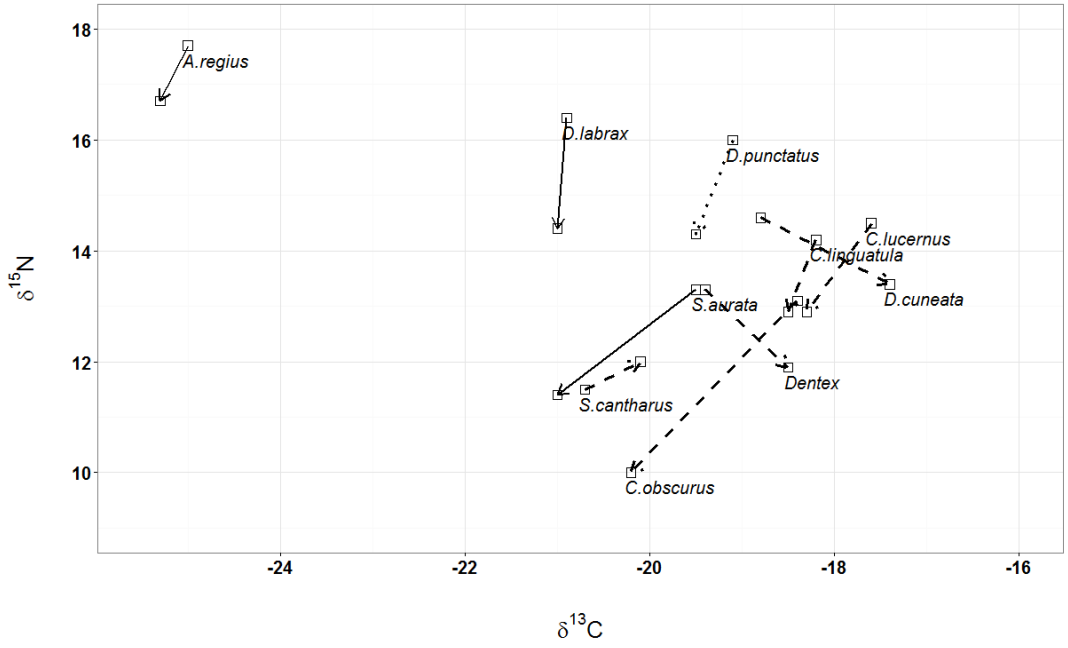

Figure 2.4.5 Spatial comparison of fish tissues stable isotopes of δ^{15}N and δ^{13}C by species. Arrows point from fish muscle to fish liver. Arrows linetype: --- Mid estuary sampling location; .... Lower estuary sampling location; - - - Offshore sampling location.

By species, a general decrease in δ^{15}N values from fish muscle to fish liver occurred with a decrease in δ^{13}C values from fish muscle to fish liver.

Only two Offshore species *Dentex* sp. and *D. cuneata* increased their δ^{13}C values with the decrease in the δ^{15}N values. Mid estuary caught *S. aurata* had intermediate stable isotopic values suggesting mix feeding, i.e. coastal waters and estuarine waters (Figure 2.4.5).

2.4.4 Correlations for FATM and SI for fish liver and fish muscle

Four Fatty Acid Trophic Markers of carnivory were correlated between fish muscle and fish liver: 18:1(n-9), $p<0.01$; 20:4(n-6), $p<0.001$; 22:6(n-3), $p<0.05$; 18:1(n-9)/18:1(n-7), $p<0.05$; PUFA/SFA, $p<0.05$. The diatom diet indicator, 16:1(n-7), was also correlated between tissues ($p<0.05$) as were the stable isotopes $\delta^{15}N$ ($p<0.001$) and $\delta^{13}C$ ($p<0.05$).

Fatty acids and fatty acid trophic markers (FATM) of fish muscle and fish liver, for all grouped samples, had generally no correlation with stable isotopes. The exceptions were: the carnivory marker 18:1(n-9)/18:1(n-7) of fish muscle that correlated with $\delta^{15}N$ ($p<0.05$) and Δ ($p<0.05$), and the diatom based marker $\sum C_{16}/\sum C_{18}$ of fish liver that correlated with Δ ($p<0.05$).

Trophic position (Δ) showed no relationship with other fatty acids or FATM. The copepod consumption FATM in fish liver 20:1(n-9)/22:1(n-9/11) correlated with $\delta^{13}C$ ($p<0.01$). The only negative correlation was between the carnivory marker 20:4(n-6) in fish muscle with $\delta^{13}C$ ($p<0.05$).

2.5 Discussion

Spatial variability in diet quality in estuaries and adjacent coastal areas challenges knowledge of the trophic dynamics in fish food webs (Pasquaud et al. 2008) and affect the small-spatial distribution of species (Leakey et al. 2008a). This study associates estuarine and coastal fishing areas with diet quality using fatty acid profiles and stable isotope composition of fish tissues.

Fish diets associated with a high assimilation of green algae (asclepic acid, 18:1(n-7)) and diatoms (EPA) in the Offshore area (Dalsgaard et al. 2003). EPA less evident in fish

liver, opposed a FATM of diatoms, palmitoleic acid, 16:1(n-7) and a FATM of copepods, oleic acid, 18:1(n-9). Herbivorous calanoid copepods, a link between primary producers and fish, use palmitoleic acid in the major pathway of fatty acids biosynthesis (Dalsgaard et al. 2003), which increases the importance of this fatty acid in the fish food web. Thus, these data showed copepods transfer the fatty acids of primary producers to higher levels in the food chain.

Fatty acids peaked for carnivory trophic markers (El-Sabaawi et al. 2009) with prevalence for consumption of copepods and dinoflagellates. If predominant fatty acids in the fish prey are DHA, palmitic acid and oleic acid, then fatty acid signatures of captured fish, reflected those of their prey (Stowasser et al. 2006) and, ultimately, of autotrophic sources (Bouillon et al. 2011). If higher levels of DHA in fish tissues reflect autotrophic sources, then data show water composition was dinoflagellate dominated, also supported by the low levels of 16:1(n-7)/16:0 (diatom indicator) (Dalsgaard et al. 2003).

Sample delipidification evened up $\delta^{13}C$ of fish muscle and depleted $\delta^{13}C$ of fish liver as higher lipid contents in fish liver lead to lower $\delta^{13}C$ (Pinnegar and Polunin 1999). Pooling by sizes was necessary to get enough material for analysis as financial and biological constraints restricted seasonal sampling that would allow to estimate short term dietary shifts. Thus, this study missed seasonal and annual variation in fatty acids and stable isotopes of energy sources via the fish tissues.

In fish, liver is a regulatory tissue with constant protein turnover, whereas muscle is a tissue of growth and affected by consumer physiological phases of growth and reproduction (Perga and Gerdeaux 2005). Tissues metabolic turnover rate, when high, respond greatest to diet shifts, e.g. liver, and represent recent feeding periods and when low, e.g. muscle, represent less recent feeding periods (MacNeil et al. 2005, Logan et al. 2006, Buchheister and Latour 2010). Nevertheless, muscle detects better diet shifts with greater power as liver exhibits higher variance among individuals in time due to internal factors that affect dietary enrichment (Sweeting et al. 2005). Seasonally, $\delta^{15}N$ and $\delta^{13}C$ of the liver provide a better assessment of the isotope

composition of the food in the field than muscle tissue (Perga and Gerdeaux 2005), including plankton, which may vary 20‰ during the year (Zohary et al. 1999).

The analysis of the fatty acid profiles and $\delta^{15}N$ and $\delta^{13}C$ suggested spatial differences of feeding sources. Temporal analysis, unrelated to specific season or year, was possible with the intra-individual comparison in the stable isotope composition of fish liver and fish muscle and detected diet shifts. A decrease in $\delta^{15}N$ from muscle to liver indicated a change in feeding behavior and/or a decrease in the trophic level of predation. This decrease conjugated with a decrease in DHA, in the mid estuary, supported a diet shift from an intense carnivory to a more omnivore feeding. Thus, a variation between fish tissues indicated diet variability at the habitat-scale.

Fractionation rates up to 20‰ in consumers increase the differences in $\delta^{13}C$ among areas but distinct $\delta^{13}C$ of energy sources are most likely the main justification for differences in the fish tissues among areas. Critical is if isotopic gradients between tissues are major when compared to isotopic variability in food sources and its effects on isotopic differences between tissues (Leakey et al. 2008a).

High levels of FATM of carnivory are typically associated with higher $\delta^{15}N$ and higher trophic level (Nyssen et al. 2005). A consistency in the mean values of the $\delta^{15}N$ of food sources and in FATM of carnivory, justifies the between-tissue correlation of these FATM and $\delta^{15}N$. Lack of other correlations indicates temporal variability in $\delta^{15}N$ in fish food. No correlation between FATM and SI disagree with studies of (Nyssen et al. 2005, Alfaro et al. 2006), e.g. increased contributors of copepods FATM 18:1(n-9), a trophic marker of carnivory, was not correlated with $\delta^{15}N$ within tissues.

Isotopic turnover defined as the isotopic change due to growth and metabolic tissue replacement is associated with a change in diet (MacAvoy et al. 2001) but mostly to tissue type (Sweeting et al. 2005). Variation among fishes, within tissue, was large and to interpret this in relation to feeding history, it is necessary to account for variation among individuals that result from metabolic differences (Leakey et al. 2008b). The standard deviation in $\delta^{13}C$ and $\delta^{15}N$ reported in this study for liver, was much higher, up to 8 times the one reported by (Sweeting et al. 2005). That reflected differences in

diet among individuals (Leakey et al. 2008b) and the unequal power to detect diet shifts by $\delta^{13}C$ and $\delta^{15}N$ analyses of different tissues (Sweeting et al. 2005).

The trophic level estimate of consumers needs a baseline $\delta^{15}N$ for estuarine and coastal food webs. This baseline can be long-lived primary consumers, as snails and mussels, captured at the same period as the fish. These will integrate isotopic changes at a spatial and temporal scale that contribute to the fish isotopic composition and quantify the trophic position of fish (Post 2002). The estuary $\delta^{15}N$ values of suspended particulate matter, when very high, as mentioned by (Machás et al. 2003) and reflected in the $\delta^{15}N$ values of consumers, if exclusively analyzed as the primary energy source, will mislead the estimate of trophic relationships. Plants decomposition material and urban sewage are potential contributors to suspended organic material, which in this case was very enriched in $\delta^{15}N$, +8.75‰ (Machás et al. 2003). Treated sewage, enriched in $\delta^{15}N$ due to microbial processes (Leakey et al. 2008a) possibly affected estuarine fishes. High $\delta^{15}N$ of Mid estuary fish, very distinct from other species, may relate to consumption of prey dependent on highly enriched estuarine material or just reflect interspecific features of feeding preferences. This way, suspended material was, in general, unimportant for fish nutrition and avoided as baseline signature and not feasible to calculate trophic position of fish.

Insight into the estuarine fish production dependency on terrestrial vascular plant material and location of fisheries production zones (Fry 2002) is provided by characteristic stable isotopic signatures of food sources of terrestrial and marine origin. Terrestrial producers are depleted in $\delta^{13}C$ and enriched in $\delta^{15}N$ relative to marine producers (Owens 1987) so the natural pattern along the estuarine-marine environment is depletion of $\delta^{13}C$ values and enrichment of $\delta^{15}N$ (Fry 2002), which were evident in this study.

The isotopic signatures of small estuarine invertebrates, predominant prey of benthivores fish, often reflect the availability of estuarine material and its strong terrestrial influence. The signatures acquired by predators reflect the signatures of their prey, the tissue analyzed, the trophic step fractionation and the turnover time of predator tissue (Tieszen et al. 1983a). Fish increasingly reflect isotopic signatures of

the migration site, which is consistent with estuarine contributions more evident in tissues from mid estuary fish (Leakey et al. 2008b).

The $\delta^{13}C$ values reflects the opportunistic use of estuarine prey by fish captured in mid estuary, while offshore fish were supported by coastal production seen in the enriched $\delta^{13}C$ that reflect small estuarine contribution to their tissue composition (Bouillon et al. 2011).

$\delta^{13}C$ values reflect overall diet and an estuarine environment and a marine environment were clearly separated based upon $\delta^{13}C$ values of fish tissues. In the estuarine environment, a broad range of $\delta^{13}C$ values suggest fish energy sources with different $\delta^{13}C$ values, either because of different sources with different $\delta^{13}C$ values or the same source with $\delta^{13}C$ values variable in time. In the marine environment, the narrow range of $\delta^{13}C$ values indicated very similar values of $\delta^{13}C$ of food sources through time (Connolly 2003b, Leakey et al. 2008a). Microphytobenthos and seagrasses (enriched $\delta^{13}C$) were assumed as a preferred common carbon source in fish food webs from the estuary and from offshore (Bouillon et al. 2011). C_3 plants and terrestrial and estuarine material (depleted $\delta^{13}C$) were only found in individuals captured in the middle of the estuary. $\delta^{13}C$ values in fish tissues suggested constant $\delta^{13}C$ values of food sources within areas but spatially distinct. Typically estuarine phytoplankton $\delta^{13}C$ values are -35‰ to -25‰, and are thus a possible energy source for estuarine fish, where $\delta^{13}C$ was about -23‰. Marine phytoplankton with $\delta^{13}C$ values from -22‰ to -20‰ and microphytobenthos with $\delta^{13}C$ values from -23‰ to -12‰ are possibly energy sources for coastal fishes where $\delta^{13}C$ values were about -19‰ (Bouillon et al. 2011).

$\delta^{13}C$ of fish tissues reflect autotrophic sources (Connolly 2003b) and despite these estuarine and coastal environment enhance fish food sources mobility and promote fish migration, due to inexistent physical barriers between the estuary and ocean and the long sampling (two years), $\delta^{13}C$ values separated well the areas and sustained non-migration. Fish often migrate between coastal and estuarine waters but stable isotope data suggest otherwise. Strong and consistent relationships in the fish tissue isotopic signatures between areas indicate low mobility of fish. Estuarine depleted $\delta^{13}C$ fish

tissues reflect a trophic affinity with the estuarine environment while coastal fish use marine energy sources, that is, no migration to estuarine areas for feeding purposes (Leakey et al. 2008a). A new diet with a different isotopic background will equilibrate animal tissues as a result of growth of new tissue and tissue metabolic turnover (Phillips and Eldridge 2005). Fish caught in Mid-estuary were resident for several months, long enough for tissues to reflect local food sources composition. Terrestrial energy sources were not a cause for an eventual migration upriver and were irrelevant as energy source for fish food web in the estuary as FATM of terrestrial organic matter were of minor abundances in fish tissues and $\delta^{13}C$ values were considerably higher than the typical very depleted values of C_3 plants (Bouillon et al. 2011).

Small differences due to metabolism or overall diet, especially if organism is omnivorous, influence the fatty acid and stable isotopes composition where the wide range of dietary quality overlap (El-Sabaawi et al. 2009).

Estuarine organic matter is unimportant for the marine benthic fish food webs. Shifts in river flow will probably not affect trophic processes in the commercial fisheries trophic webs in coastal waters but estuarine species will be affected due to their dependency on local autotrophic sources.

Fish opportunistic feeding behavior and the wide range of seasonal diet choice justify for the wide range in $\delta^{13}C$ and $\delta^{15}N$ values for fish tissues. Thus, the unlikely equilibrium of marine fishes with their diet (MacNeil et al. 2005). Offshore fish fit a benthivore invertebrate-dominated diet with a narrower range of isotopic values compared to mid estuary fish, which fit a mixed diet of piscivores and benthivores (MacNeil et al. 2005).

Trophic position estimates of fish need $\delta^{15}N$ values, validated $\delta^{15}N$ fractionation values and dietary data of the entire food web, and a reliable baseline. Fractionation values among tissues obtained from controlled feeding experiments and detailed stable isotopes sampling of all possible fish food sources (MacNeil et al. 2005) are preferred than the long-established and widely used but variable 3.4‰ fractionation in $\delta^{15}N$ (Post 2002).

Via the contribution of terrestrial and marine organic matter in the fish food webs, temporal and spatial series of δ^{15}N and δ^{13}C and FATM detect hydrological events that affect unusual upstream migrations of fishes and, ultimately, the local economy (Connolly 2003b).

Knowledge on trophic pathways from autotrophic sources to fisheries species challenges the common studies of biodiversity and elucidates on temporal and spatial effects of energy sources on the imperative end-point, fisheries productivity (Leakey et al. 2008a).

3 Terrestrial organic matter input in coastal fish food web

3.1 Abstract

The energy sources analysis of valuable coastal fisheries species include terrestrial organic matter from vascular plants transported by river flow. The terrestrial to marine connectivity is evaluated by the importance of terrestrial organic matter in the trophic web of these fishes. This incorporation was measured in fish from Guadiana estuary and adjacent coastal areas with fatty acid composition for fish muscle tissue and particulate and sedimentary organic matter and stable isotopic composition of nitrogen and carbon for fish muscle, in 2010 and 2011. $\delta^{15}N$ and $\delta^{13}C$ values for fish showed high interspecific variability, most relevant in Mid estuary. Stable isotopes suggested a contribution of material from terrestrial origin at the base of the fish food web. The low levels of fatty acids trophic markers typical of vascular plants excluded these as relevant contributors for fisheries species nutrition. The methods complemented the conclusion on the low contribution of terrestrial matter from vascular plants in fisheries species food webs in Guadiana estuary and adjacent coastal areas and thus, of the weak terrestrial influence in marine fish food web. This study further suggested that marine organic matter in the estuary occurred from upstream migration of coastal fisheries species. This movement of organic matter within the estuary is susceptible to environmental change with consequences to freshwater inflow and thus, on the connectivity between marine, estuarine and terrestrial habitats.

3.2 Introduction

Autotrophic sources in many aquatic systems, sustain the trophic webs from the bottom to the top in complex and unclear pathways. Estuaries and coastal areas are, among others, nursery (Kostecki et al. 2010) and feeding grounds for fish (Connolly et al. 2005b) where organic matter transported by river flows stimulate productivity (Bunn and Arthington 2002, Kostecki et al. 2010, Botto et al. 2011) but evidence on the magnitude of the effect of terrestrial matter as autotrophic source for fisheries species is limited (Able 2005). Coastal fish biomass is supported by zooplankton (including larval fish) that depends directly on terrestrial or estuarine matter or via estuarine phytoplankton (Loneragan et al. 1997, Connolly et al. 2005b, Schlacher et al. 2009).

Commercial fisheries in the Guadiana estuary adjacent areas reached landings of a net value up to 3.5 million Euros in 2014, the second largest in the Algarve region (INE 2015). Sustainability of fisheries habitat and fisheries productivity in the Guadiana adjacent coastal zone needs awareness and protection of fish estuarine habitat and respective terrestrial energy sources habitats (Connolly et al. 2005b, Abrantes and Sheaves 2009). Trophic relationships remain elusive for fish species in this estuary with fish diet characterized by (Sá et al. 2006) with stomach contents. The link between terrestrial organic matter to estuarine and coastal fisheries species, including the spatial segregation between autotrophic sources and fisheries species is understudied for this estuary. Likely, energy sources of coastal fisheries species, captured in the estuary, rely somehow on terrestrial matter supplied by river flow.

The ecosystem direct response to river flow and land-use change, if measured, will enhance nearshore fisheries productivity estimates (Bunn and Arthington 2002).

Trophic tracers unravel nutrient pathways and energy sources in complex trophic webs linked to estuaries (Peterson and Fry 1987). Fatty Acids Trophic Markers (FATM) are diet-based tracers, characteristic of certain marine species or groups and conserved along the food chain and not biosynthesized by the fish (Sargent 1976). FATM, such as

linoleic acid (*cis*-9,12-octadecadienoic acid; 18:2(n-6)) derives from terrestrial matter, specifically from agriculture and vascular plants; oleic acid (*cis*-9-octadecenoic acid; 18:1(n-9)) generally indicates carnivory on copepods or derives from brown algae in estuarine environment. Oleic acid is also a precursor of arachidonic acid, ARA (cis-5,8,11,14-eicosatetraenoic; 20:4(n-6)), the most important metabolite from fatty acid biosynthesis in marine algae and indicates typical carnivory. α-linolenic acid (*cis*-9,12,15-octadecatrienoic acid; 18:3(n-3)) derives from salt marsh and vascular plants and palmitoleic acid (*cis*-9-hexadecenoic acid; 16:1(n-7)) is found in high concentrations in diatoms. Linoleic acid, 18:2(n-6), and α-linolenic acid, 18:3(n-3), are found only in primary producers, (Graeve et al. 2001, Auel et al. 2002, Dalsgaard et al. 2003, Alfaro et al. 2006, Kelly and Scheibling 2012).

Combination of several fatty acids indicates the presence of particular algal classes as primary producers of the trophic web. A ratio between the monosaturated fatty acid palmitoleic acid, 16:1(n-7) and the saturated fatty acid, palmitic acid (hexadecanoic acid, 16:0), 16:1(n-7)/16:0>1, indicates dominance of diatoms (bacillariophytes) at the base of the trophic web (Auel et al. 2002, Dalsgaard et al. 2003). The sum of linoleic acid, 18:2(n-6) with α-linolenic acid, 18:3(n-3), 18:2(n-6)+18:3(n-3), indicates terrestrial matter originated from vascular plants incorporated via the trophic web (Budge and Parrish 1998). The sum of gondoic acid (*cis*-11-eicosenoic acid, 20:1(n-9)) with erucic acid (*cis*-13-docosenoic acid, 22:1(n-9)) and cetoleic acid (*cis*-11-docosenoic acid, 22:1(n-11)), 20:1(n-9)+22:1(n-9/11), indicates carnivorous feeding on copepods. Copepods are unique to biosynthesize *de novo* considerable amounts of monosaturated fatty acids with 20 and 22 carbon atoms, e.g. herbivorous copepods biosynthesize large quantities of gondoic acid, 20:1(n-9) and cetoleic acid, 22:1(n-11) from one step elongation of oleic acid, 18:1(n-9) and gadoleic acid (cis-9-eicosenoic acid), 20:1(n-11)), respectively (Dalsgaard et al. 2003).

Fatty acids become less clear with increasing trophic levels (Auel et al. 2002) and changes in the fatty acid composition also relate to time of year, sex, geographic location, specific tissue under investigation (MacNeil et al. 2005). Asclepic acid (*cis*-11-octadecenoic acid; 18:1(n-7)) is most abundant in green algae and also a precursor of cetoleic acid, 22:1(n-11).

High value in the ratio of EPA or timnodonic acid (*cis*-5,8,11,14,17-eicosapentaenoic acid; 20:5(n-3)) - abundant in diatoms - and DHA (*cis*-4,7,10,13,16,19-docosahexaenoic acid; 22:6(n-3)) - abundant in dinoflagellates - EPA/DHA, reveals diatom over dinoflagellate dominance as primary producers in the food web. The ratio $\sum C_{16}/\sum C_{18}$ is similar to EPA/DHA, since C_{16} fatty acids are more abundant in diatoms and C_{18} more abundant in dinoflagellates, (Budge and Parrish 1998, Auel et al. 2002, Dalsgaard et al. 2003, Kelly and Scheibling 2012).

Stable isotope analysis complement fatty acids analysis to describe food sources (Kelly and Scheibling 2012). Consumers, at whatever trophic level, reflect the isotope ratio of energy sources, though with fractionation shift, especially for nitrogen (Peterson 1999). The trophic levels are evaluated with stable isotopes of nitrogen ($\delta^{15}N$) and energy sources with stable isotopes of carbon ($\delta^{13}C$) (DeNiro and Epstein 1981, Fry 2006). $\delta^{15}N$ increases 3-5‰ per trophic level (Hobson 1990) and at the species level, $\delta^{15}N$ estimates the number of trophic links between food source and consumers (Peterson 1999), e.g. terrestrial plants (-5‰ to 1‰, marine algae (1‰ to 7‰) (Romanuk and Leavings 2005).

$\delta^{13}C$ increases 1‰ stepwise in the food chain. This estimates the distance of a predator to ultimate energy source, the primary producer (Michener and Schell 1994, Kelly and Scheibling 2012). Different isotopic ratios of $\delta^{13}C$ discriminates primary producers, e.g. terrestrial producers are $\delta^{13}C$ depleted when compared to marine producers, terrestrial plants have -33‰ to -22‰ while marine algae have -20‰ to -10‰ (Romanuk and Leavings 2005).

Consumers isotope ratios vary due to trophic position and spatial and temporal changes in the isotopic compositions at the base of the food web (Peterson and Fry

1987). Thus, analyses of isotopic composition may associate transfer of terrestrial produced material to benthic food webs (Peterson et al. 1985, Zeug and Winemiller 2008) and infer if the fish are feeding on terrestrial or marine prey (Romanuk and Leavings 2005).

This study aimed to evaluate the link of riverine terrestrial organic matter from vascular plants to fish captured in the Guadiana estuary and adjacent coastal area. The primary hypothesis was if fish, captured inside the estuary, feed primarily in the estuarine area, then organic matter of terrestrial origin would be a main energy source. Tracers would then reveal the importance of terrestrial matter from vascular plants in estuaries for fish nutrition.

3.3 Materials and methods

3.3.1 Study site

The Guadiana estuary is a mesotidal system, in the Southern Portugal bordering Spain (37º11'N, 7º25'W) that opens to the north-western Gulf of Cádiz, as shown in Figure 3.3.1 (Barbosa et al. 2010). Its catchment basin is the fourth largest in the Iberian Peninsula, approximately 67.500 km^2 (Chícharo et al. 2006). This estuary is 70 km long: Mértola defines the high estuary where the salinity is close to zero but with tidal influence; the middle estuary, ca. 38 km upstream, is a transition zone where the water is brackish most of the time; the low estuary, with a salinity similar to seawater, has two main habitats: the salt marsh and the main river channel (Chícharo et al. 2006, Sá et al. 2006, Barbosa et al. 2010).

Hydro-sedimentary processes in this estuary affect downstream fish habitats, upstream migration behavior and estuarine trophic dynamics. These will constrain

estuarine and coastal fisheries production, and examples are sediment loading, reduction of estuarine plume that lowers the cue for coastal marine fish to migrate upstream to spawning and nursery areas or nutrients exported downstream (Morais 2008).

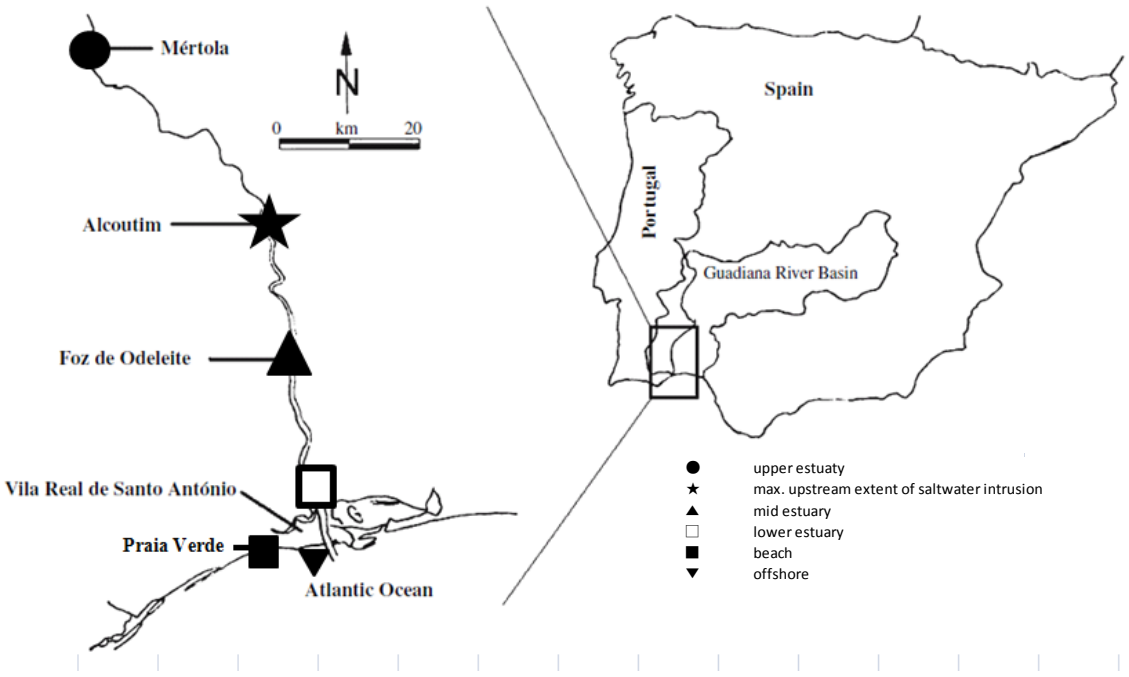

Figure 3.3.1 Map of the sampling sites for POM and fish in the Guadiana estuary, coast and in offshore the river plume (adapted from Domingues et al. 2011).

The temperate climate of the region, under Mediterranean influence, is characterized by freshwater flow with pronounced seasonal and yearly variability, usually with lower freshwater flow, higher temperatures and salinities in the summer months (Cravo et al. 2006, Morais 2008). The water temperature is between 10ºC in the winter and 28ºC in the summer. Monthly rainfall fluctuates between 0 mm per month in summer and intermittent major rainfall events that reach above 200 mm per month (Barbosa et al. 2010).

The seasonal pattern of the river drainage was modified by a recent dam, 150 km upstream, which increased freshwater retention up to 81%. Pulse water discharges became more frequent in the winter and reduced river flow occurs in the summer (Morais et al. 2009).

3.3.2 Sample collection

3.3.2.1 Organic matter

POM (suspended particulate organic matter) and SOM (sediment organic matter) was collected in summer 2011, at three sites within the Guadiana estuary: Upper estuary, Mértola; Mid estuary (Odeleite); Lower estuary (river mouth); and a coastal beach (Praia Verde) 7 km west of the river mouth.

Freshwater flow from the Guadiana River and oceanic water from the Atlantic Ocean affected the sites salinity differently. Mértola was only influenced by freshwater (salinity: 0.19±0.01), as the maximum upstream saltwater intrusion is Alcoutim, 40km downstream; Odeleite was most influenced by freshwater (salinity: 0.21±0.02); the river mouth was intermediate between the two water masses (salinity: 22.14±4.64); and the coastal beach was outside the river outflow influence (salinity: 34.28±1.59) (Figure 3.3.1). Samples, three bottles of water and three sediment samples, were taken at the same time of the day in June for POM and SOM and September for POM. Sediment samples were collected by scrapping the top 1-2 cm off the surface layer of the sediment.

At the laboratory, each water sample was pre-filtered through a 200µm mesh to remove zooplankton and detritus, then filtered on pre-combusted (500ºC for 4h) GF/C glass microfiber filter under moderate vacuum. The sediment was mixed in filtered water from the same location and sieved through a 200µm to remove detritus and

larger particles, then filtered on a pre-combusted (500ºC for 4h) GF/C glass microfiber filter under moderate vacuum.

3.3.2.2 Fish samples

Fish were collected from three areas: Mid-estuary of the Guadiana River; Lower estuary of the Guadiana River and Offshore (12-15 nm from the Guadiana River plume) between autumn 2010 and summer 2011 (Figure 3.3.1). The fish were captured with different gears: long lines, gillnets and hooks.

Immediately after sampling, individuals were dissected to extract muscle tissue. Muscle was chosen over liver as the fatty acid composition of muscle tissue reflects former diet over a longer period of time, compared to liver tissue which reflects latter feeding (Stowasser et al. 2006).

When available, tissues from individuals of the similar size were pooled. Pooled samples were composed of tissues of two or three individuals, when available, captured in the same site and time. Samples were stored frozen at -20ºC prior to biochemical analyses.

3.3.3 Sample analyses

3.3.3.1 Fatty acids analyses

Fatty acid composition in fish was determined for 27 samples of fish tissues, of which n=17 samples were composed of two or three individuals of the same size (Table 3.4.1). POM and SOM filters of the same location and date were assembled in one sample to obtain enough matter to analyze fatty acid composition.

Lipids were isolated from samples of fish muscle and organic matter filters using a modification of the method of (Folch et al. 1957). Butyl hydroxyl toluene was added to the samples and lipids were extracted using a chloroform-methanol solvent (2:1 v/v) mixture. The organic layer was separated by centrifugation and the extract evaporated to dryness by rotary evaporation. An appropriate amount of lipid extract (10mg) was transferred into clean test tubes or diluted in 1mL distilled toluene if the lipid is not visible.

The lipids were converted into fatty acid methyl esters (FAMEs) by transesterification overnight (incubated at 50^{o}C) by adding 1mL of distilled toluene and methanol-containing sulphuric acid (1% v/v) to each tube and homogenizing the mixture. The methyl esters were extracted into *iso*-hexane and dried over anhydrous sodium sulphate at -20^{o}C until analysis. The extracts were diluted to 1mg/mL.

Trans-esterified lipids were analyzed by gas chromatography with flame ionization detection (GC-FID) using an Agilent (Hewlett-Packard) 5890 Series II gas chromatograph, equipped with a cool, on-column auto injector, fitted with a fused silica capillary column (0.25mm i.d. x 30m) coated with a 0.25µm film of 50% cyanopropyl. The extracts were injected (1µL) at 60^{o}C. The oven temperature was ramped at 25^{o}C/min from 60^{o}C to 150^{o}C and then 1^{o}C/min to 200^{o}C. The temperature was held constant for 10 minutes before final elevation at 50^{o}C/min to 230^{o}C where it was held for 5 minutes. The detector was set at 300^{o}C. Nitrogen was used as the carrier gas (1mL/min) (Webster et al. 2006). The data was collected and processed using Perkin Elmer Turbochrom Navigator 6.1.0.2:G07 software (Perkin Elmer, Beaconsfield, UK).

Laboratory reference materials were ran with the samples for appropriate assurance checks to confirm GC retention times and as a check on the esterification process and procedural blanks were also ran with each batch of samples. Twenty-nine fatty acid methyl esters were identified based on the retention times of the laboratory

standards. Results are expressed as normalized area percentage, i.e. percentages of the total area of the 29 fatty acids.

The input of terrestrial matter organic sources in the fish diet was deduced from the relative abundance of typical FATM in the fish muscle tissue fatty acid profile. Oleic acid, 18:1(n-9) may indicate the input of salt marsh plants; linoleic acid, 18:2(n-6) indicated the input of saltmarsh plants and green algae input; α-linolenic acid, 18:3(n-3) indicated the input of saltmarsh plants and vascular plants (Dalsgaard et al. 2003, Kelly and Scheibling 2012).

3.3.3.2 Stable isotopes analyses

Stable isotopes were analyzed from delipidified muscle tissue, of 18 fish samples. Delipidification was necessary as lipid contents affect $\delta^{13}C$ (DeNiro and Epstein 1977, Tieszen et al. 1983a, Nyssen et al. 2005).

Samples were kept frozen at -20°C then thawed and dried at 60°C for 24h or until constant weight. Dried samples were ground and homogenized into a powder. 0.6mg±10% of each sample was transferred into pre-weighed 6x4 mm SerCon tin capsules, sealed and kept in a dessicator until analysis.

A SerCon Carbon and Nitrogen Isotope Analyser coupled with an element analyser (IRMS, isotope ratio mass spectrometer) combusted samples and analyzed resulting CO_2(g) and N_2(g) for carbon and nitrogen stable isotope ratios.

Internal laboratory standards were run at regular intervals for every batch of samples analyzed along with randomly displaced duplicates of the samples.

Isotopic composition is expressed as Δ (‰), which is the ratio between the sample isotopic ratios and international standards reference materials isotopic ratios (Vienna Pee Dee Belemnite for carbon and atmospheric N_2 for nitrogen). Isotopic composition ratio is calculated from: $\delta R = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000$, where $R = \frac{^{13}C}{^{12}C}$ and $R = \frac{^{15}N}{^{14}N}$.

Δ estimates are relative to laboratory working standards adjusted to international standards. USGS40 is the reference material used to calibrate stable carbon ($\delta^{13}C$) and stable nitrogen ($\delta^{15}N$). $\delta^{13}C$ values are relative to VPDB (Vienna PeeDee Belemnite) (Coplen et al. 2006) and $\delta^{15}N$ values are relative to atmospheric nitrogen (Mariotti 1983). $\delta^{15}N$ isotopic measurements had a precision of <0.4‰ and $\delta^{13}C$ isotopic measurements had a precision of <0.2‰.

3.3.4 Statistical analysis

Statistical analysis was based on the identified 29 fatty acids. Minor fatty acids contributed less than 5% to the fatty acid profile and were excluded.

3.3.4.1 Multivariate analysis

Multivariate analyses of the fatty acid composition were performed for all individuals using the program R, with the packages "BiodiversityR" and package "vegan" (R Core Team 2013). No transformation was applied to the data set because those fatty acids that contribute only to a small percentage of the total composition did not feature heavily in the diet. Giving artificial weight to these minor fatty acids by applying a transformation would therefore be inappropriate (Nyssen et al. 2005).

Principal component analysis (PCA) explores relationships among samples and simplifies large fatty acids data sets.

PCA combines correlated fatty acids into new components and reduces large numbers of variables to a few components that represent most of the variance in the data. This allows identification of fatty acids that contribute most to separate groups and fatty

acids highly correlated (Budge et al. 2006). Fatty acids trophic markers (FATM) of food sources of interest help interpret PCA results if their contribution discriminates groups. The assumptions of PCA are less stringent and reliable results require more samples than variables.

Inertia was decomposed for each fatty acid and fatty acid indicator to select the most influential in the analysis and determine their correlations.

Multivariate analysis of variance (MANOVA) tested significant differences in overall fatty acid composition among groups of samples by examining whether mean differences among groups occurred by chance. The null hypothesis was that multivariate means of all groups were equal, i.e. no effect of treatment, as estuary section. MANOVA assume the data are multivariate normal and the covariance matrices are homogeneous (Kelly and Scheibling 2012).

Correlation between fatty acids and fatty acids trophic markers and indicators with higher inertia values was determined for $p<0.001$.

3.3.4.2 2 Dimension biomarker approach

Fatty acid composition analysis and stable isotope data analysis complement to detail pathways of organic matter sources. $\delta^{15}N$ and $\delta^{13}C$ values were plotted versus fatty acid biomarkers characteristic of terrestrial matter from vascular plants and saltmarsh plants (Nyssen et al. 2005).

3.4 Results and Discussion

3.4.1 Fish communities

Guadiana river freshwater discharge affected the estuarine fish community in terms of distribution and species composition in the study by (Chícharo et al. 2006). Fish species were distributed by areas: low estuary; middle estuary; and freshwater upper estuary (with abundances and biomass dominated by anchovies, barbels and *Halobatrachus didactylus*).

The reduced number of freshwater species and variability of species in the samples likely reflected less shifts in environmental conditions due to regular river freshwater flow (Chícharo et al. 2006).

The variability of species in the estuary also depends on the outflow to the coastal zone, where freshwater turbidity plumes guide coastal adult fishes into the estuary (Bunn and Arthington 2002). Thus, the presence of marine species in a mid estuary indicates either turbidity plumes or movement of seawater upstream. Table 3.4.1 summarizes fish samples analyzed: 18 species *per* year and estuary section with a total of 27 individual or pooled samples. The species captured were *Argyrosomus regius* (Asso, 1801), *Barbus* sp., *Bothus podas* (Delaroche, 1809), *Chelidonichthys lucernus* (Linnaeus, 1758), *Chelidonichthys obscurus* (Walbaum, 1792), *Citharus linguatula* (Linnaeus, 1758), *Dentex* sp., *Dicentrarchus labrax* (Linnaeus, 1758), *Dicentrarchus punctatus* (Bloch, 1792), *Dicologlossa cuneata* (Moreau, 1881), *Diplodus annularis* (Linnaeus, 1758), *Halobatrachus didactylus* (Bloch & Schneider, 1801), *Scomber japonicus* Houttuyn, 1782, *Solea senegalensis* Kaup, 1958, *Sparus aurata* Linnaeus, 1758, *Spondyliossoma cantharus* (Linnaeus, 1758), *Trachinotus ovatus* (Linnaeus, 1758), *Trachinus draco* Linnaeus, 1758.

The species caught, mostly benthic carnivores, feed on bivalves, crustaceans and other fish. The exceptions are *Scomber japonicus* that also feeds on the pelagic environment and *Sparus aurata* that is benthic omnivore (Froese and Pauly 2008). These species have generally a great interest in the local economy and are commercially captured in coastal areas.

Table 3.4.1 Summary of fish samples analyzed from the catches in the Mid and Lower Guadiana estuary and from Offshore commercial catches. The letter X represented one sample, with a single individual or a pool of individuals with the same size (n=2 or n=3) for each species, per year and estuary sampling location.

	Guadiana River estuary									
	Mid estuary				Lower estuary				Offshore	
	2010		2011		2010		2011		2011	
Species	single sample	pooled sample	single sample	pooled sample	single sample	pooled sample	single sample	pooled sample	single sample	pooled sample
Argyrosomus regius	x									
Barbus sp.		x								
Bothus podas									x	
Chelidonichthys lucernus										x
Chelidonichthys obscurus									x	
Citharus linguatula										x
Dentex sp.										x
Dicentrarchus labrax		xxx			x					
Dicentrarchus punctatus		xx					x			
Dicologlossa cuneata									x	
Diplodus annularis										x
Halobatrachus didactylus							x			x
Scomber japonicus								x		
Solea senegalensis			x							
Sparus aurata		x			x		x			
Spondyliossoma cantharus									x	
Trachinotus ovatus								x		
Trachinus draco								x		x

In a study on fish feeding ecology and trophic interactions in the lower Guadiana estuary, (Sá et al. 2006) fish assemblages were dominated by marine species: *Solea* sp., *Dicentrarchus* sp., *Mullus surmuletus*, *Diplodus* sp., *Sparus aurata*, *Liza* sp. and the most abundant *Halobatrachus didactylus*. The method of stomach content analysis concluded fish opportunistic feeding behavior on amphipods, polychaetes, small fish and shrimps, was sensitive to environmental changes in the estuary (e.g. river freshwater discharges, temperatures, input of organic material).

3.4.2 Fatty acids analysis

The contrast of autotrophs and consumers fatty acid composition highlights pathways of energy and nutrients transfer and splits single source from a combination of sources to consumers diet. The importance of primary producers (e.g. vascular plants, seagrass, epiphytic algae or/and microalgae) to marine trophic web (Melville and Connolly 2005) is recognized due to *de novo* biosynthesis of certain PUFA, as linoleic acid, 18:2(n-6) and α-linolenic acid, 18:3(n-3) which are precursors of ARA, EPA and DHA, essential to marine consumers (Dalsgaard et al. 2003).

The fish muscle fatty acid composition (Table 3.4.2) was richer in DHA (PUFA), 22:6(n-3); palmitic acid, 16:0 (SFA); and oleic acid, 18:1(n-9) (MUFA) relative to EPA, 20:5(n-3) (PUFA); stearic acid, 18:0 (SFA); ARA, 20:4(n-6) (PUFA). PUFA dominated the fatty acid composition of Offshore samples.

Estuary locations failed to affect the mean fatty acid composition of fish muscle ($p>0.1$) nor Fatty Acid Trophic Markers (FATM) typical of terrestrial input from vascular plants ($p>0.01$). The FATM: linoleic acid, 18:2(n-6) and α-linolenic acid, 18:3(n-3), were negligible in the fish muscle when compared to predominant oleic acid, 18:1(n-9); EPA, 20:5(n-3) and DHA, 22:6(n-3).

Table 3.4.2 FAME components ±1SD in fish muscle captured at Mid and Lower Estuary sections of the Guadiana and Offshore. Data were expressed as normalized percentages (those without uncertainty calculated are asterisked).

	Mid Estuary		Lower Estuary		Offshore	
	Muscle		Muscle		Muscle	
	n=9		n=8		n=10	
Fatty acid and indicators	Mean	SD	Mean	SD	Mean	SD
14:0	0.95	0.45	1.52	0.99	1.25	0.75
14:1(n-5)	0.09	0.09	0.09	0.07	0.22	0.11
15:0	0.48	0.16	0.47	0.11	0.49	0.10
16:0	20.00	2.26	19.97	3.59	19.52	2.01
16:1(n-7)	3.05	1.15	3.71	2.95	2.50	1.61
16:2*	0.15	0.15	1.02	2.41	0.35	0.27
16:3*	0.30	0.25	0.10	0.18	0.02	0.08
16:4*	0.22	0.20	0.15	0.12	0.01	0.02
17:0	0.89	0.39	2.96	6.25	0.89	0.27
18:0	7.28	0.63	6.50	1.94	8.04	1.48
18:1(n-9)	11.35	2.85	11.35	5.33	7.23	3.36
18:1(n-7)	2.72	0.53	2.38	1.20	4.35	5.07
18:2(n-6)	0.92	0.56	1.05	0.61	0.92	0.26
18:3(n-6)	0.21	0.11	0.11	0.03	0.09	0.05
18:3(n-3)	0.31	0.17	0.39	0.21	0.34	0.21
18:4(n-3)	0.09	0.09	0.04	0.08	0.23	0.20
20:0	0.34	0.30	0.22	0.13	0.30	0.11
20:1(n-11)	0.72	0.30	0.74	0.41	0.75	0.28
20:1(n-9)	0.52	0.33	0.41	0.17	0.36	0.20
20:2(n-6)	0.35	0.25	0.24	0.10	0.51	0.18
20:3(n-3)	0.24	0.13	0.16	0.08	0.17	0.10
20:4(n-6)	5.94	1.49	4.08	2.95	4.72	1.83
20:4(n-3)	0.30	0.15	0.42	0.15	0.43	0.19
20:5(n-3)	9.93	2.38	8.12	3.08	9.56	2.70
22:1(n-9/11)	0.49	0.87	0.16	0.06	0.65	0.87
21:5(n-3)	0.03	0.05	0.07	0.08	0.12	0.15
22:5(n-3)	4.23	2.06	3.85	2.67	3.94	1.44
22:6(n-3)	26.61	4.41	25.56	8.78	31.21	7.54
24:1(n-9)	1.29	2.04	4.17	8.21	0.82	0.54
SFA	29.95	2.43	31.65	6.58	30.49	1.93
MUFA	20.22	3.58	23.00	8.72	16.89	5.11
PUFA	49.68	4.92	44.34	12.51	52.27	6.28
PUFA/SFA	1.68	0.28	1.51	0.61	1.73	0.30
16:1(n-7)/16:0	0.15	0.05	0.19	0.17	0.13	0.07
18:1(n-9)/18:1(n-7)	4.35	1.48	6.17	4.09	2.62	1.11
18:2(n-6)+18:3(n-3)	1.2356	0.5898	1.445	0.7985	1.262	0.4639
20:1(n-9)+22:1(n-9/11)	1.01	0.9666	0.5738	0.2085	1.013	0.9455
20:5(n-3)/22:6(n-3)	0.38	0.12	0.32	0.08	0.33	0.16
22:6(n-3)/20:5(n-3)	2.86	0.98	3.31	1.05	3.62	1.65
SumC16	23.71	3.08	24.94	5.75	22.41	3.02
SumC16/SumC18	1.04	0.12	1.23	0.37	1.08	0.18
SumC18	22.88	2.72	21.82	7.02	21.20	3.82

The higher concentration of DHA (Figure 3.4.1) compared to EPA suggested a predominance of dinoflagellate over diatoms in the fish diet.

Oleic acid, 18:1(n-9) concentrations suggested a substantial input of organic matter from direct or indirect consumption of copepods or estuarine brown algae, e.g. (Dalsgaard et al. 2003, Kelly and Scheibling 2012).

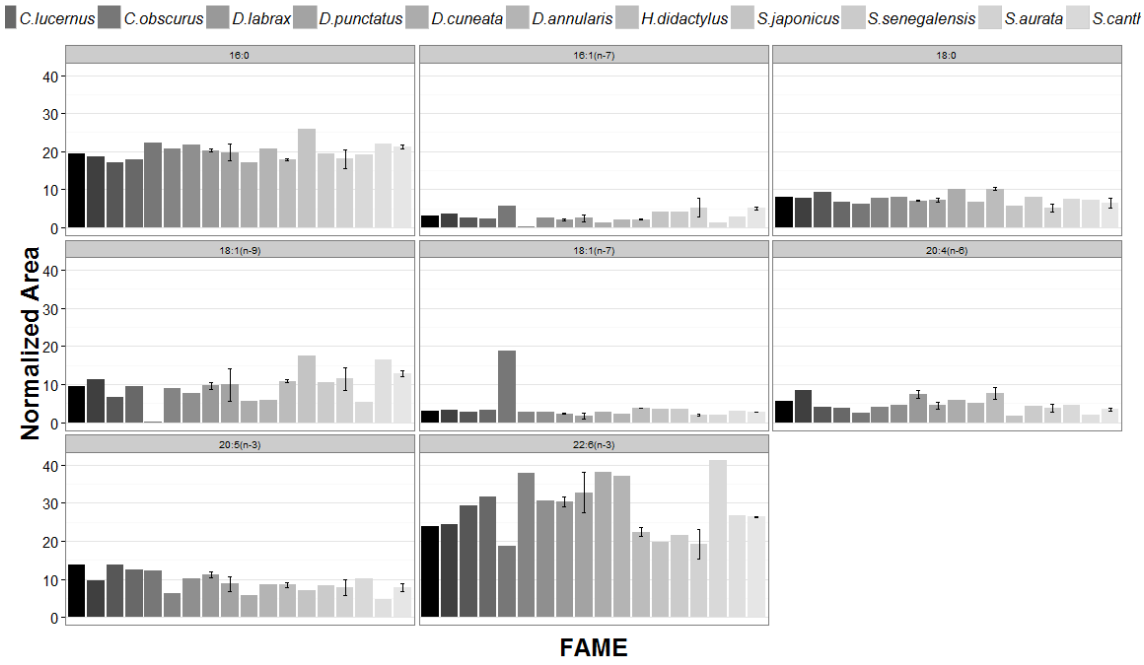

Figure 3.4.1 Major fatty acids of fish muscle. Selected fatty acids represented concentrations higher than 10% or relevant for terrestrial input assessment. Data were expressed as mean of normalized area percentage(%)+ standard error.

The fatty acids trophic markers most abundant in the particulate organic matter (POM) and sedimented organic matter (SOM) (Figure 3.4.2) are common in: diatoms, 16:1(n-7); green algae, 18:1(n-7); terrestrial matter from vascular plants, 18:2(n-6) and 18:3(n-3); and copepods, 20:1(n-11) and 22:1(n-9/11). In this case, POM and SOM sampled along the estuary, throughout the year, had a fatty acid composition inconsistent with terrestrial matter from vascular plants as main contributors.

POM and SOM exceeded fish muscle for 20:1(n-11), 18:1(n-7) and mainly, the *de novo* copepod biosynthesized, 22:1(n-11) (Dalsgaard et al. 2003). Fatty acids from copepod origin dominated the POM and SOM.

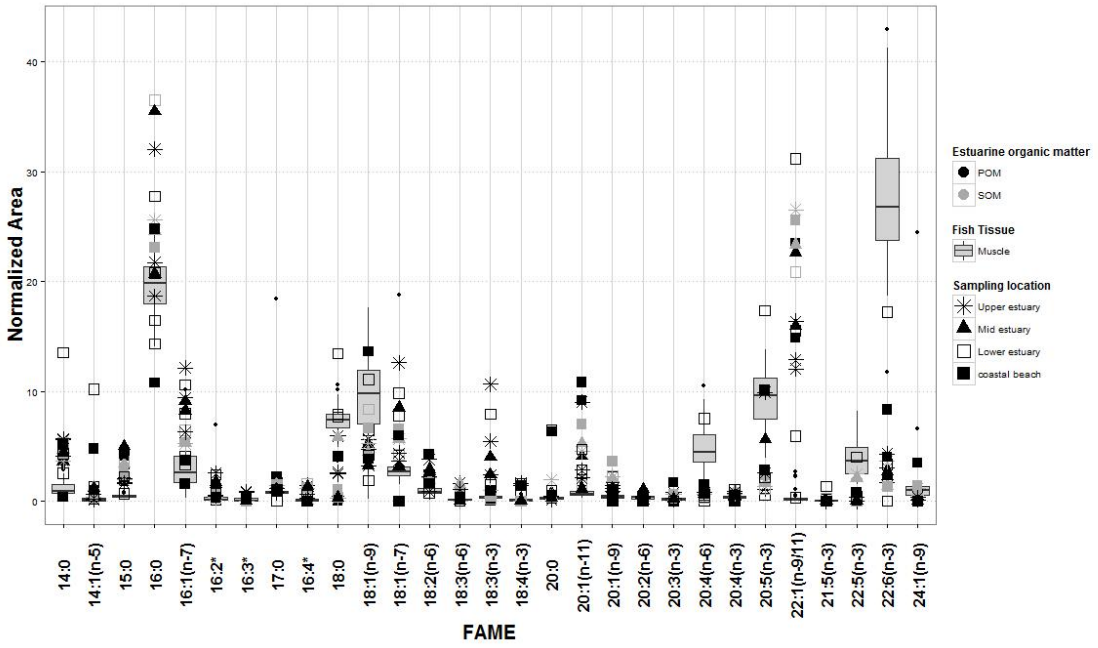

Figure 3.4.2 Boxplots represented FAME normalized percentage area in samples of muscle tissue of fish (n=27) captured in the Guadiana Mid Estuary, Lower Estuary and Offshore. Points represented normalized percentage area of samples of Sediment Organic Matter (SOM) and Suspended Particulate Organic Matter (POM) in the Upper, Mid and Lower estuary and at the Beach (Praia Verde Beach, 7 km east from the River mouth) for all sampling occasions. (Fame uncertainty calculated are asterisked).

Based on FATM concentrations, terrestrial matter from vascular plants transported by the Guadiana river flow lacked as a nutrition source of fisheries species in the Guadiana estuary. This result contrasted with the expected trophic subsidies of POM carbon transported by rivers for detrital food webs in estuarine and coastal food webs (Schlacher et al. 2009, Connolly et al. 2009).

3.4.2.1 Multivariate analysis of fatty acids composition

Oleic acid, 18:1(n-9), asclepic acid, 18:1(n-7) and DHA, 22:6(n-3) represented most of the data variance and contributed greatly to sample separation (Figure 3.4.3). ARA, 20:4(n-6) results from the elongation and desaturation from oleic acid, 18:1(n-9) which justifies their high correlation ($p<0.01$). EPA was highly correlated with its low abundant precursor α-linolenic acid, 18:3(n-3) ($p<0.05$) (Dalsgaard et al. 2003).

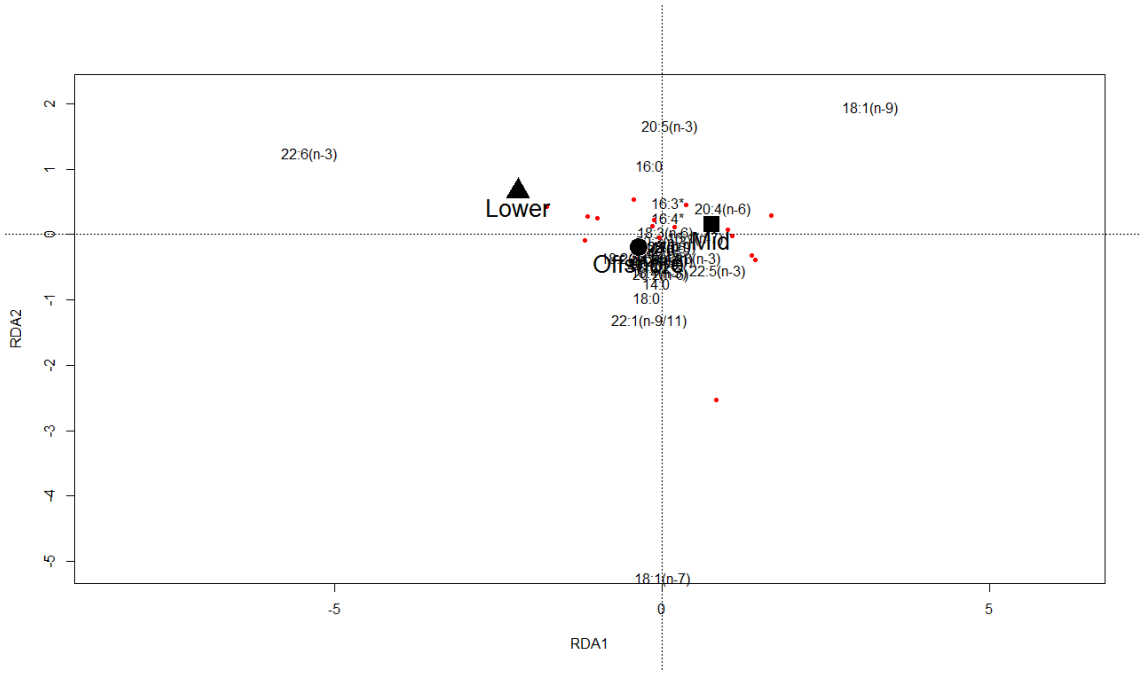

Figure 3.4.3 Principal component analysis of fatty acid composition (normalized percentage area) of fish muscle (n=27) constrained to sites of fish sampling in Guadiana Mid Estuary and Lower Estuary and Offshore.

3.4.3 Stable isotope analysis

3.4.3.1 Nitrogen fish isotope values

Nitrogen isotope ratios ($\delta^{15}N$) showed significant spatial contrasts (p=0.0018) between Mid Estuary (mean= 16.06‰, sd=1.78‰, min=13.32‰, max=18.86‰, n=7) and Offshore (mean=13.31‰, sd=1.19‰, min=11.42‰, max=14.58‰, n=9). Differences (Figure 3.4.4) ranged up to more than 3‰ in the Mid Estuary. The single sample from Lower Estuary had a value of $\delta^{15}N$ =16.02‰, intermediate of Mid Estuary and Offshore samples.

Isotopic fractionation varies among species (MacAvoy et al. 2001) and without isotopic baseline, $\delta^{15}N$ values were inadequate to determine trophic levels. The usual trophic enrichment (+2‰ to +4‰ per trophic level) between consumers and their presumed food and the $\delta^{15}N$ values of all fish samples (11.42‰ to 18.86‰) indicated organic matter sources with $\delta^{15}N$ values higher than 6‰.

Typically, terrestrial organic matter $\delta^{15}N$ values are under +3‰ (Bouillon et al. 2011), which presumes the irrelevance of this energy source. This exemplifies $\delta^{15}N$ as an indicator for the lack of C3 plants in the base of the food web for C3 plants $\delta^{15}N$=+0.4‰±0.9‰; *Spartina alterniflora* $\delta^{15}N$=+6.0‰±2.1‰ and plankton $\delta^{15}N$=+8.6‰±1.0‰, as mentioned in (Peterson 1999).

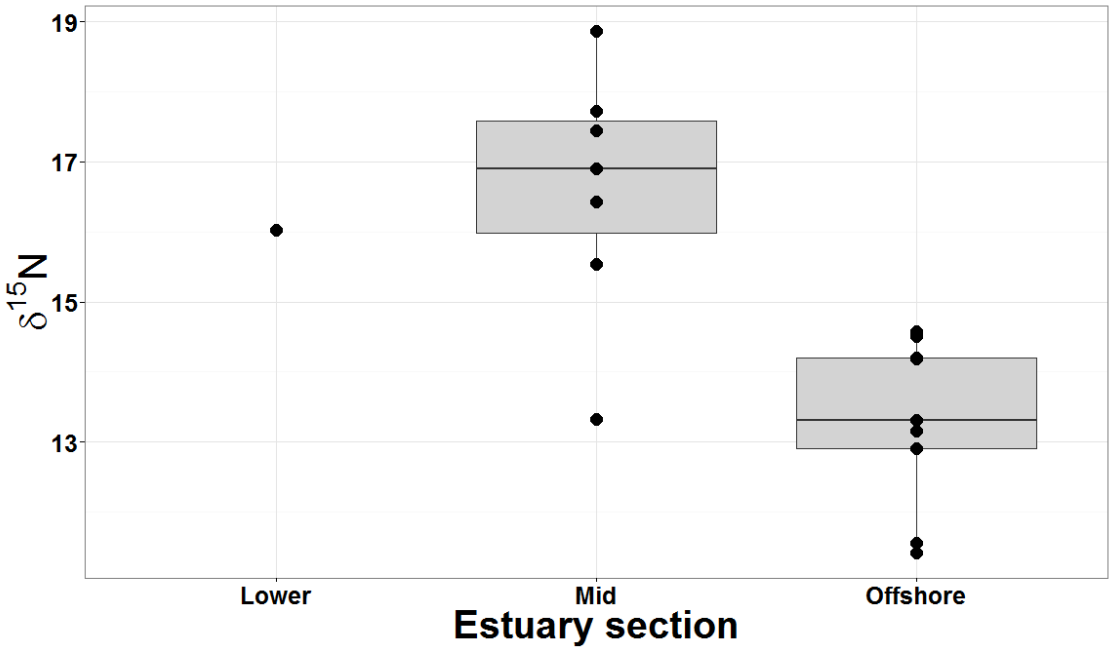

Figure 3.4.4 Boxplots of $\delta^{15}N$ of fish muscle from Guadiana Mid estuary, Lower estuary and Offshore.

3.4.4 Terrestrial nutrition sources for fish

$\delta^{13}C$ slightly enriches with trophic transition (about 1‰) and can allow to follow organic matter sources in food webs (Pasquaud et al. 2008). In this study, doubts arose in the origin of the carbon source to fish due to similar $\delta^{13}C$ values of estuarine and coastal particulate organic matter (Kostecki et al. 2010).

$\delta^{13}C$ differed among sites ($p<0.05$) with depletion of mean carbon isotopic ratios and increase in isotopic variability with upstream distance from the sea (Figure 3.4.5). Carbon isotope depletion was up to 4.09‰ in Mid Estuary fish (mean $\delta^{13}C$=-23.15‰, sd=3.22‰, min=-26.23‰, max=-19.06‰) compared to Offshore fish (mean value of $\delta^{13}C$ =-19.06‰, sd=1.03‰, min=-20.66‰, max=-17.59‰).

The spatial differences in the fish $\delta^{13}C$ values suggested different sources of organic matter as, generally, consumers assimilate local organic matter. The influence of riverine terrestrial organic matter was suggested where $\delta^{13}C$ values were strongly depleted, lower than -25‰ (Peterson 1999, Bouillon et al. 2011). The Offshore fish were the most enriched in $\delta^{13}C$ values. Lower Estuary fish were also markedly enriched ($\delta^{13}C$ =-19.09‰) when compared to some Mid Estuary fish. Presumably, more enriched $\delta^{13}C$ values derived from organic matter sources produced by marine phytoplankton stimulated by marine nutrients (Deegan and Garritt 1997, Connolly et al. 2009, Bouillon et al. 2011). Within Mid Estuary, fish with very depleted $\delta^{13}C$ values (-25.04‰ to -26.23‰), when compared to the Offshore fish lower value ($\delta^{13}C$ =-20.66‰), presumably had a greater uptake of estuarine and terrestrial organic matter discharged by freshwater flow (Bouillon et al. 2011).

Enriched $\delta^{13}C$ values in the fish muscle were, presumably due to low consumption of terrestrial matter transported by river flow and/or higher supply of enriched $\delta^{13}C$ organic matter (Kostecki et al. 2010).

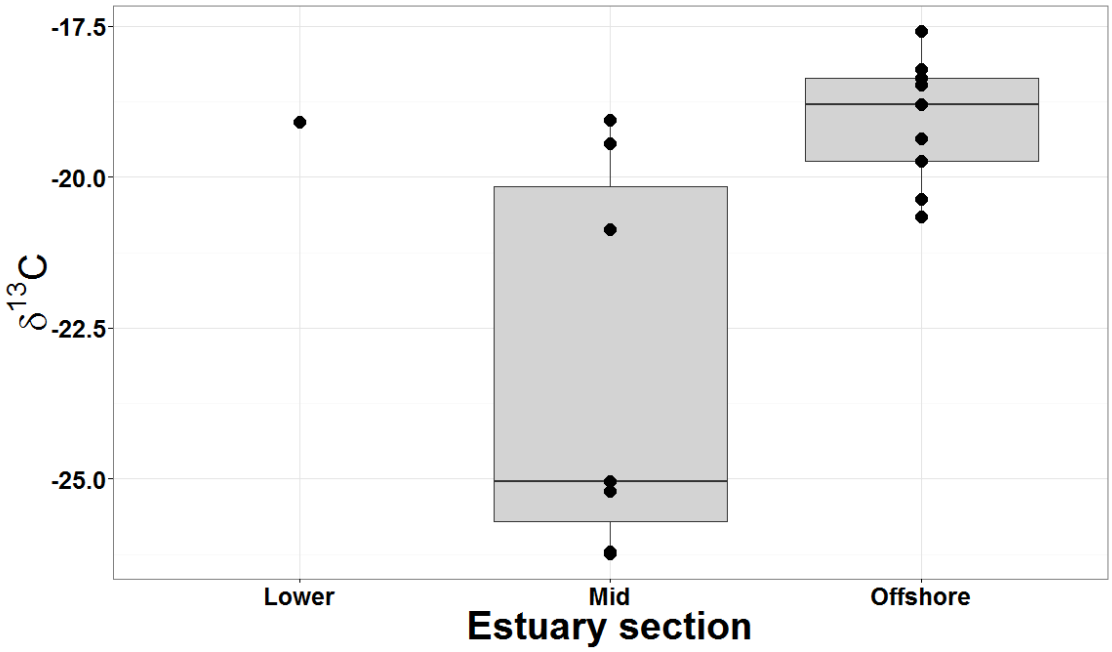

Figure 3.4.5 Boxplots of $\delta^{13}C$ of fish muscle from Guadiana Mid estuary, Lower estuary and Offshore.

Higher interspecific variability for $\delta^{13}C$ values of fish within the Mid Estuary suggest various sources of organic matter supply the base of the trophic web for each fish. The most depleted $\delta^{13}C$ values arrayed between estuarine oligohaline phytoplankton (-35‰ to -20‰) and C3 plants (typical of terrestrial environment, -30‰ to -25‰). The most enriched $\delta^{13}C$ values indicated greater influence of microphytobenthos (-23‰ to -12‰), marine phytoplankton (-22‰ to -20‰), C4 plants (-15‰ to -12‰) and/or seagrasses (-20‰ to -10‰) (Bouillon et al. 2011).

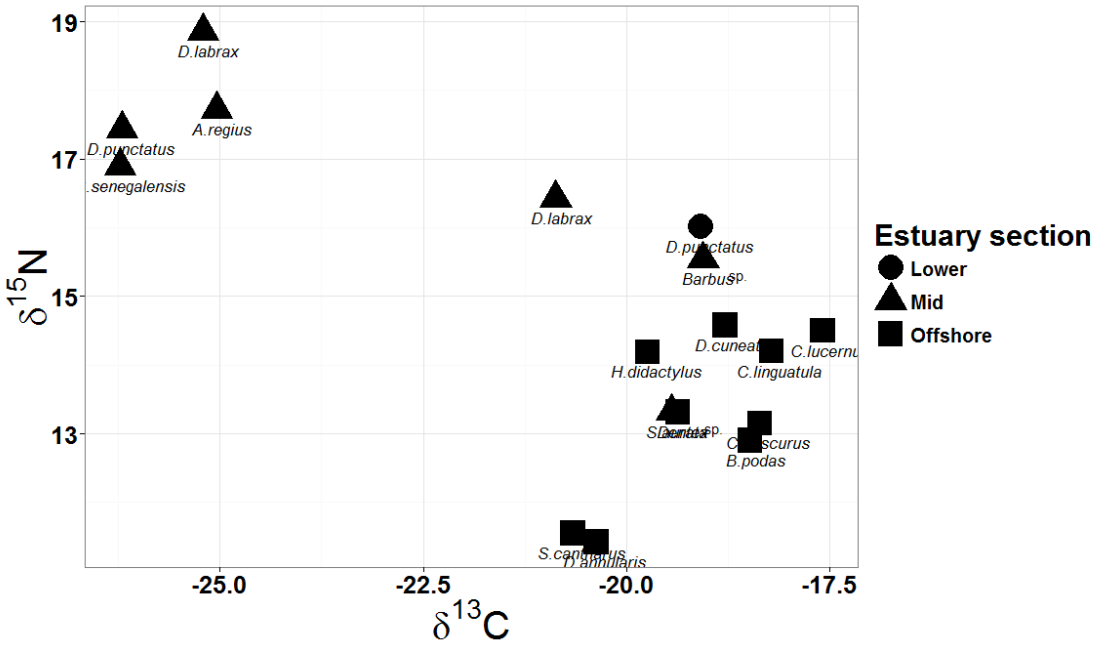

Figure 3.4.6 $\delta^{15}N$ versus $\delta^{13}C$ for muscle tissue of fish captured in Mid estuary, Lower estuary and Offshore.

One group of fish segregated in the Mid Estuary (Figure 3.4.6) with high $\delta^{15}N$ and low $\delta^{13}C$. This indicated a higher trophic level for these fish in the food web (e.g. carnivory) where, presumably, carbon derived from prey sustained by depleted $\delta^{13}C$ food, either of terrestrial origin or locally produced.

Offshore, the stable isotope ratios and the negative correlation between $\delta^{13}C$ and $\delta^{15}N$ ($p<0.001$) suggested a feeding mode of omnivory dependent on enriched $\delta^{13}C$ energy sources, as marine microphytobenthos (Riera and Richard 1997, Bouillon et al. 2011).

3.4.5 2 Dimension biomarker approach

The combination of fatty acid and stable isotope data is useful to trace organic matter for fish at different trophic positions (e.g plot of $\delta^{15}N$ values versus fatty acid biomarkers).

The sum of terrestrial input fatty acid biomarkers, linoleic acid (18:2(n-6)) and α-linolenic acid (18:3(n-3)) indicated the input of organic matter derived from terrestrial matter, specifically agriculture and vascular plants and salt marsh plants (Budge and Parrish 1998, Kelly and Scheibling 2012). This indicator low values may result from the synthesis of major fatty acid end products (ARA, EPA, DHA) from these fatty acids (Dalsgaard et al. 2003) or an irrelevant contribution of vascular plants to the fish nutrition.

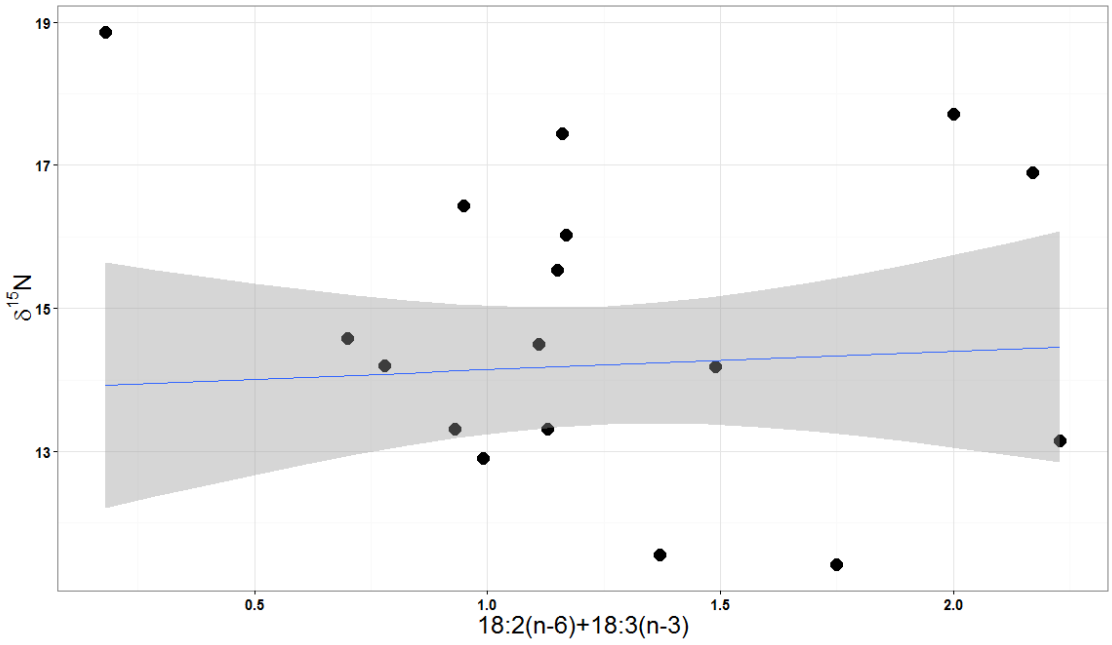

Figure 3.4.7 $\delta^{15}N$ versus bioindicator of terrestrial organic matter in diet (18:2(n-6)+18:3(n-3)) in fish tissues.}

$\delta^{15}N$ was not correlated to 18:2(n-6)+18:3(n-3) ($p>0.1$) (Figure 3.4.7), thus the rank of fish in the food web was independent of the terrestrial matter FATM concentration. For instance, carnivore fish may use an organic matter from terrestrial origin, if feeding on prey sustained by terrestrial organic matter, while coastal omnivore fish may feed offshore on marine organic matter and migrate upstream.

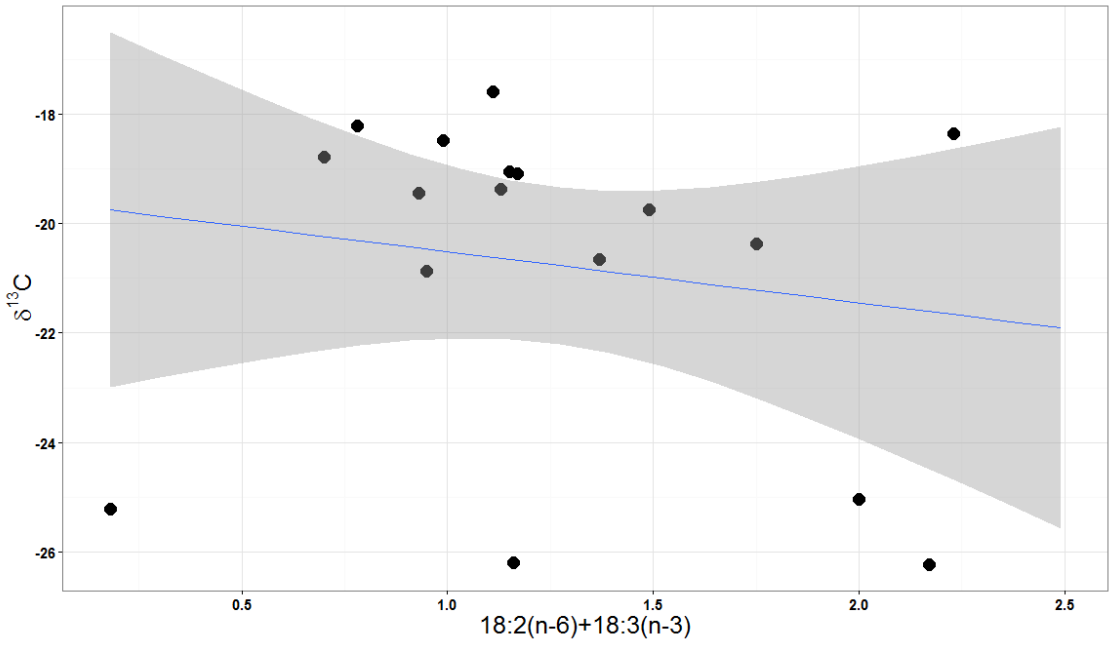

Figure 3.4.8 $\delta^{13}C$ versus bioindicator of terrestrial organic matter in diet (18:2(n-6)+18:3(n-3)) in fish tissues.

$\delta^{13}C$ values of fish depend on the distance from the base of the food web, i.e. number of trophic transfers, and $\delta^{13}C$ values of primary producers at the base of the food web (Michener and Schell 1994, Romanuk and Leavings 2005). The fish energy sources included two groups of values: enriched $\delta^{13}C$ organic matter, coincident with marine primary producers and/or higher number of trophic levels; and depleted $\delta^{13}C$ organic matter, coincident with terrestrial matter. The low values (<2.5%) for the fatty acid bioindicator 18:2(n-6)+18:3(n-3) for all fish muscle samples strengthened the unimportance of terrestrial matter from vascular plants as major source of nutrition for these fisheries species (Figure 3.4.8).

Though this bioindicator and $\delta^{13}C$ were not correlated ($p>0.5$), depleted $\delta^{13}C$ values of fish muscle indicated diet input from depleted sources. The low values of this bioindicator justified estuarine phytoplankton and microphytobenthos as contributors for the depleted $\delta^{13}C$ values and not vascular plants. Organic matter from marine system, enriched in $\delta^{13}C$ was presumably a major source of nutrition in these fish.

The movement of carbon was, thus, negligible to promote terrestrial matter from vascular plants as a trophic subsidy to fisheries species in the estuary and adjacent coastal waters though Guadiana estuary is connected directly to coastal waters, i.e. nonexistent barrier islands (Connolly et al. 2005a, Abrantes and Sheaves 2009). The unimportance of outwelled terrestrial detritus to support species in coastal habitats was previously shown by Lee (1995).

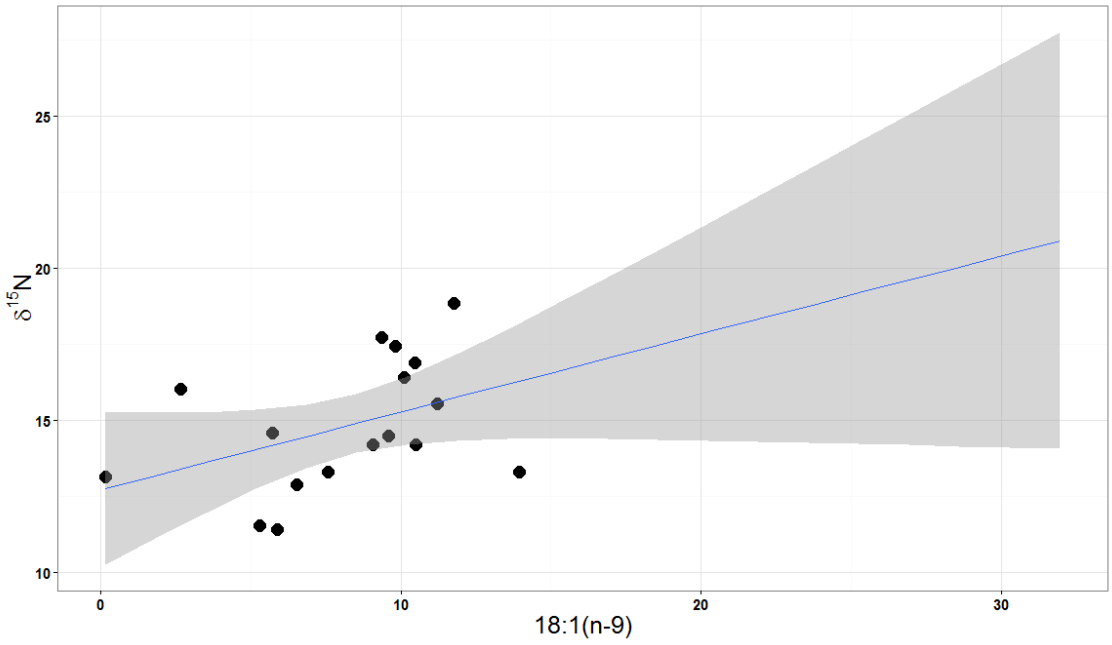

Figure 3.4.9 $\delta^{15}N$ versus bioindicator of carnivorous diet or input of brown algae (normalized area of 18:1(n-9)) in fish tissues.

Oleic acid, 18:1(n-9), independent of the variation of $\delta^{15}N$ or $\delta^{13}C$ ($p>0.05$) showed a positive tendency with the rank of species in the trophic web ($\delta^{15}N$). This suggested an accumulation of this fatty acid with trophic links (Figure 3.4.9). Thus, the concentration of oleic acid indicated the level of carnivory rather than the contribution of brown algae as an organic matter source at the base of the food web.

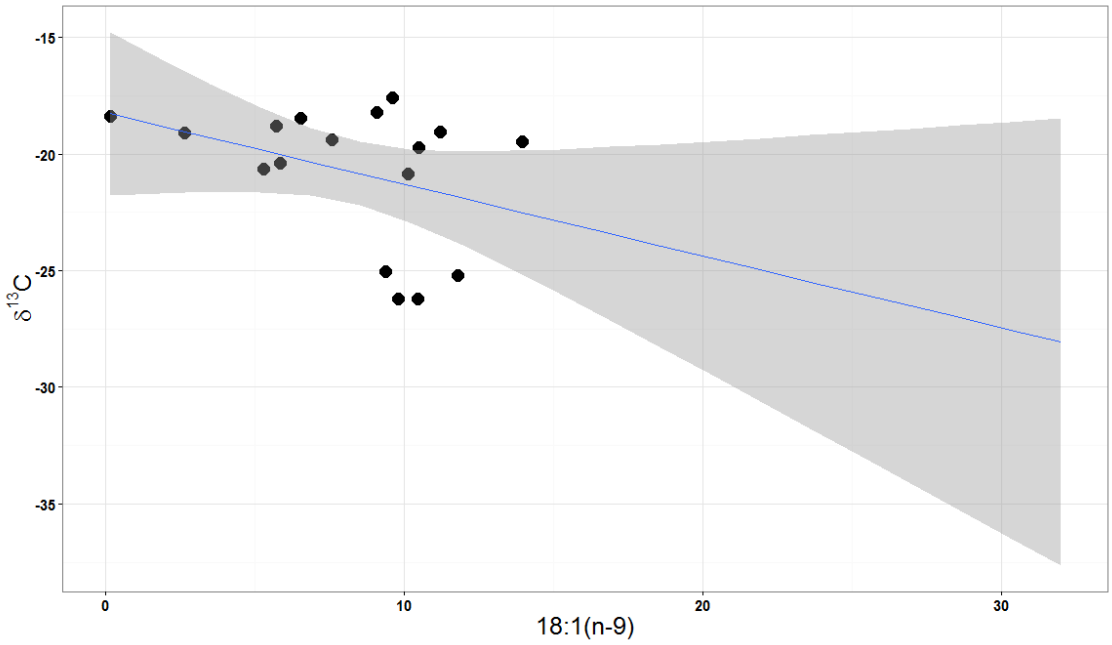

Figure 3.4.10 $\delta^{13}C$ versus bioindicator of carnivorous diet (normalized area of 18:1(n-9)) in fish tissues.

The negative tendency between oleic acid, 18:1(n-9), and $\delta^{13}C$, suggested depleted $\delta^{13}C$ energy sources for several carnivorous fish (Figure 3.4.10).

High values of this fatty acid, typical of carnivory, may result from copepod consumption along the food web. Copepods selectively feed on high quality phytoplankton in turbid estuarine environments and reject low quality non-living matter, as lithogenic particles and detritus (e.g. vascular plants material) which are the bulk of the suspended matter pool (Connolly et al. 2009). Nevertheless, enriched $\delta^{13}C$ values in fish muscle may result from terrestrial and estuarine organic matter when is incorporated via microbial and microzooplankton trophic links. This way isotopic changes associated with material processing are less detectable (Schlacher et al. 2009).

3.5 Conclusion

This study provides the first proof of lack of contribution of organic material from vascular plants to fisheries species nutrition in Guadiana estuary and nearshore coastal areas. Fatty acids analysis complemented stable isotopes analysis to detail the unimportance of vascular plants as energy source.

The spatial segregated terrestrial autotrophic sources (Riera and Richard 1997, Connolly et al. 2005a, Schlacher et al. 2009) in the Guadiana river, connects with estuarine and marine fish by reduced continuous and strong pulsed freshwater flows (Cravo et al. 2006). Nevertheless, marine phytoplankton resources were a most probable and abundant source of energy fish in the estuary and adjacent coastal areas (Bunn and Arthington 2002, Le Pape et al. 2003, Schlacher et al. 2009). These findings are supported by FATM (Bunn and Arthington 2002, Darnaude 2005, Kostecki et al. 2010) and enriched $\delta^{13}C$ values in the fish muscle that presume feeding on benthic marine areas from food sources as microphytobenthos and epiphyte microalgae, available via predation on invertebrates (Connolly et al. 2005a, Melville and Connolly 2005, Schlacher et al. 2009). Besides, FATM excluded vascular plants from fish trophic links to terrestrial matter, suggested by depleted $\delta^{13}C$ values in fish muscle (Budge and Parrish 1998).

The movement of organic matter within the estuary is susceptible to freshwater inflow and thus, on the connectivity between the estuarine and marine habitats.

Designing estuarine protected areas must consider trophic links between nearshore and estuarine habitats (Schlacher et al. 2009) via organic matter supply to the base of fish food webs (Melville and Connolly 2005). Future work would achieve tighter resolution of the relative contributions and sources of organic matter to the fisheries species food webs and examine spatial and temporal variation in the fish. For example, compound-specific stable isotopes would separate clearly the contribution of marine phytoplankton from estuarine organic matter and terrestrial matter (Evershed et al. 2007). This work is the precursor to more rigorous statistical testing of spatial correlations between organic matter transported by Guadiana river flow and fisheries species location of capture.

4 Stable isotope from multiple tissues of the cephalopod *Octopus vulgaris*: delipidification implication

4.1 Abstract

Multiple-tissue analyses achieve more information on feeding relationships than a one-tissue analysis. Isotopic enrichment depends on the turnover rate of each tissue. Tissues with higher turnover rates integrate diets on a shorter period than tissues with a lower turnover rate. However, inter-tissue variability may confound results as tissues have different lipid composition and fractionation. $\delta^{13}C$ is a useful tracer of primary carbon sources, however is negatively correlated to content of lipids in tissues since lipids are depleted in carbon isotopic ratios compared to protein. To evaluate the effect of lipids in the stable isotopes used as trophic biomarkers in the *Octopus vulgaris*, delipidified and non-delipidified tissues were compared for tissues with similar composition and function (mantle and tentacles) and for tissues with higher contents in lipids. Greater differences were found for $\delta^{13}C$ of digestive gland compared to mantle, with more depleted values in the non-delipidified tissues. This study demonstrates delipidification is essential prior to isotopic analysis for inter-tissue comparisons in the *O. vulgaris*, to obtain better resolution in isotopic differences; and mantle and tentacle are similar in isotopic compositions. The careful application of stable isotope methods to several tissue types is a powerful tool to investigate diets and to use benthic organisms as ecological indicators of environmental changes. Lipids should be extracted prior to stable isotope analysis to avoid bias in $\delta^{13}C$ measurements due to different lipid contents. The effect of lipids in $\delta^{15}N$ is not significant.

4.2 Introduction

Cephalopods are important consumers in marine food webs with increasing importance in the worldwide catches (Xavier et al. 2015). Large fluctuations occur in cephalopods populations and a lack in dietary studies affect productivity resource management. The use of stable isotopes analyses trophic interactions is a common method nowadays in trophic dynamics for several purposes (MacNeil et al. 2005).

Differences occur among tissues of a single cephalopod and between the same tissue among individuals in a small distance and that must be considered for trophic studies and the variation in stable isotope composition is pertinent in trophic studies.

Even methodological procedures are relevant in the final results of the stable isotopic composition. The removal of lipid from the samples, since lipid synthesis depletes $\delta^{13}C$ (DeNiro and Epstein 1977, Bodin et al. 2007) provides potential sources of variability not associated with the source or the tissue.

The objectives are to understand fractionation in different tissues of the cephalopod *Octopus vulgaris* to reduce variability in $\delta^{13}C$ attributable to factors other than diet isotopic variability, plus, to evaluate the effect of $\delta^{13}C$ depletion due to lipids composition in tissues. It is expected that the lipid removal has significant effects on isotopic ratios in tissues with high lipid contents.

4.3 Materials and methods

The species *O. vulgaris* was selected due to its high commercial value and economical importance to small scale fisheries in the Algarve region and the potential and currently unpredictable fluctuations in the productivity (Sonderblohm et al. 2014). Octopuses were dissected on the fishing day. Tissues extracted were: part of the mantle and tentacle, entire digestive gland, and gut contents (later homogenized). The sampled tissues were kept frozen at -20ºC until analysis.

4.3.1 Stable isotopes analyses

Stable isotopes were analyzed from mantle, digestive gland, tentacle and gut contents, of 33 octopuses. Tissue samples (mantle, digestive gland and gut) were delipidified from the fatty acids analysis and were kept frozen at -20ºC until analyses.

Tissue samples of mantle, digestive gland and tentacle were not delipidified and were kept frozen at -20ºC until analyses. After thawing, samples were oven dried at 60ºC for 24h or until constant weight, then ground and homogenized with a mortar and pestle into a fine powder and weighted to 0.6mg±10% into pre-weighed 6x4mm SerCon tin capsules. Folded and compacted capsules were kept in a labeled tray in a dessicator until further analysis.

For isotopic composition measurement, samples were combusted in a SerCon Carbon and Nitrogen Isotope Analyser coupled with an element analyser (IRMS, isotope ratio mass spectrometer). Several samples ran in duplicate at random and internal laboratory standards ran at regular intervals for every batch of samples analyzed. Resulting CO_2(g) and N_2(g) were analyzed for carbon and nitrogen stable isotope ratios.

Isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) expressed in a δ notation as per thousand (‰) difference between isotopic ratios in the sample and international standards reference

materials (Vienna Pee Dee Belemnite for carbon and atmospheric N_2 for nitrogen), were calculated with the formula:

$$\delta R = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000$$

where $R = \frac{^{13}C}{^{12}C}$ or $R = \frac{^{15}N}{^{14}N}$, i.e. *R* measured the ratio of heavy to light isotope in the sample. Values were raw mass spectrometry δ estimates relative to laboratory working standards, adjusted to international standards. USGS40 was the reference material measured in an elemental analyser (EA) to calibrate stable carbon (δ^{13}C) and nitrogen (δ^{15}N) of unknown carbon and nitrogen-bearing substances (Qi et al. 2003). Stable carbon isotopic values were relative to VPDB (Vienna PeeDee belemnite) (Coplen et al. 2006) and stable nitrogen isotopic values were relative to atmospheric nitrogen, which is isotopically homogeneous (Mariotti 1983). Results had a precision of <0.4‰ for δ^{15}N and <0.2‰ for δ^{13}C.

4.3.2 Statistical analysis

Pairwise t-tests analyzed for the effect of delipidification on stable isotope values of δ^{15}N and δ^{13}C in the mantle and digestive gland); and the isotopic routing in δ^{15}N and δ^{13}C in non delipidified tentacle and mantle tissues.

All statistical analyses were performed using R software (R Core Team 2013). Mean values were calculated ±SD.

ANOVA tested for the effect of small distances of sampling on the isotopic signatures through the comparisons of δ^{15}N and δ^{13}C in the non-delipidified tissues, through the replicates comparisons.

4.4 Results

Delipidification had an effect on $\delta^{15}N$ and $\delta^{13}C$ values for tissue types, digestive gland and mantle of the cephalopod *O. vulgaris*. Intra-tissue $\delta^{15}N$ differences were most evident for mantle with higher values for delipidified mantle. $\delta^{13}C$ values of both delipidified tissues were significantly lower than for non-delipidified (Table 4.4.1).

Table 4.4.1 Pairwise t-test for the treatment delipidification of tissues (digestive gland, mantle) for the cephalopod *O. vulgaris* in the $\delta^{15}N$ and $\delta^{13}C$ isotopic signatures. Means and SD of $\delta^{15}N$ and $\delta^{13}C$ values are represented for each tissue and treatment.

Treatment: delipidification		
	$\delta^{13}C$	$\delta^{15}N$
Digestive Gland	p<0.0001	p<0.05
Mantle	p<0.0001	p<0.0001

Means ± SD		
	$\delta^{13}C$	$\delta^{15}N$
Digestive Gland		
delipidified	-18.46±1.53	8.34±0.67
non-delipidified	-20.37±1.40	8.68±1.03
Mantle		
delipidified	-15.89±0.74	10.65±0.71
non-delipidified	-17.22±1.29	9.56±0.71

O. vulgaris $\delta^{15}N$ values of non-delipidified mantle was, on average, 4.5‰ greater than in tentacle ($p<0.0001$). For $\delta^{13}C$, the non-delipidified mantle and tentacle were not significantly different.

$\delta^{15}N$ and $\delta^{13}C$ isotopic values in digestive gland was lower than for mantle and tentacle, for both treatments, when applicable (Figure 4.4.1 and Figure 4.4.2).

The replicate effect was slightly significant for $\delta^{15}N$ and $\delta^{13}C$ isotopic signatures of tentacle and digestive gland tissue ($p<0.05$), both non-delipidified.

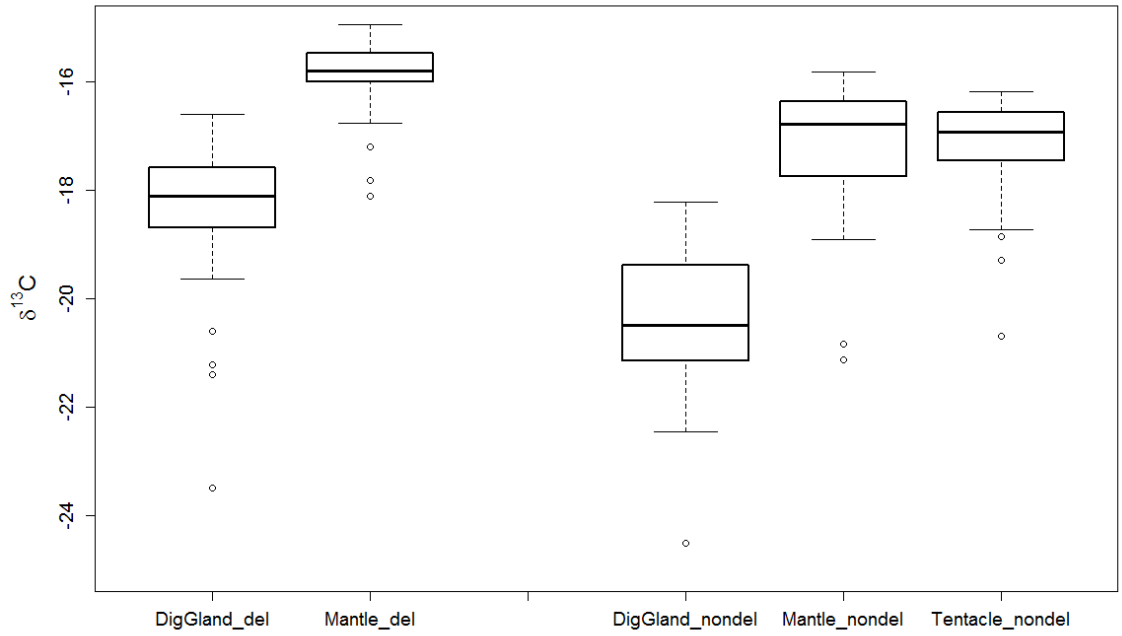

Figure 4.4.1 Boxplots of $\delta^{13}C$ isotopic signatures for tissues of *O. vulgaris* (DigGland: digestive gland, Mantle: mantle, Tentacle: tentacle) conditional on treatment delipidification for tissues digestive gland and mantle (del: delipidified; nondel: non-delipidified).

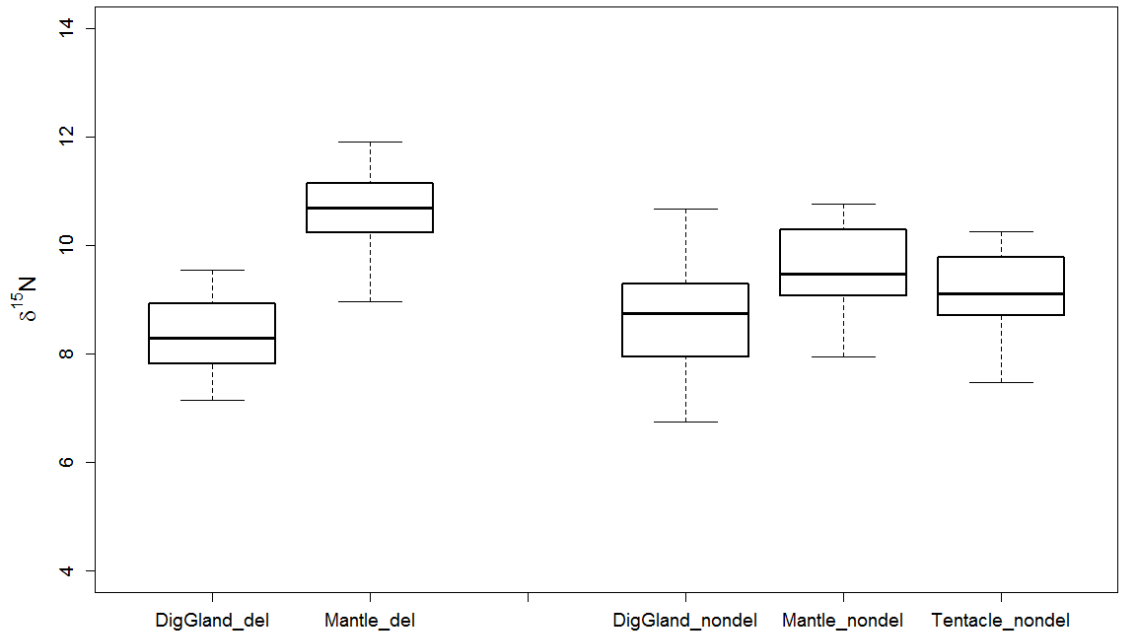

Figure 4.4.2 Boxplots of $\delta^{15}N$ isotopic signatures for tissues of *O. vulgaris* (DigGland: digestive gland, Mantle: mantle, Tentacle: tentacle) conditional on treatment delipidification for tissues digestive gland and mantle (del: delipidified; nondel: non-delipidified).

4.5 Discussion

This study demonstrated differences in the isotopic signatures among three studied tissues (non-delipidified), muscle tissues: mantle and tentacle; and digestive gland of the cephalopod *O. vulgaris*. The isotopic signatures between these muscle tissues were similar despite a small difference in the $\delta^{15}N$ but with very close values for $\delta^{13}C$. Digestive gland was much depleted in $\delta^{13}C$ compared with the muscle tissues (Deudero et al. 2009, Cherel et al. 2009b).

Spatial variability was evaluated with samples from seven replicates and a weak significance ($p<0.05$) was demonstrated for tentacles and digestive gland for both stable isotopic values, on non-delipidified tissues. This suggests that the response in

the different tissues depend on the locations where these cephalopods were captured. Thus, limitation of sampling in a restricted area at only one time is at risk of misleading conclusions (Deudero et al. 2009).

Nitrogen fractionation is expected to have a larger variation in coastal and marine animals (up to 5.8‰), especially when feeding different diets (Yokoyama et al. 2005). However, isotopic values showed low deviations for all tissues. The difference for $\delta^{15}N$ values between all the non-delipidified tissues is lower than 1‰ and for $\delta^{13}C$ is lower than 3‰.

Differences in isotopic values between the studied tissues can be resultant from the tissue specific turnover rates (Gannes et al. 1998, MacAvoy et al. 2001). Muscle tissues, mantle and tentacle, showed higher isotopic values than digestive gland. These tissues have lower turnover rates than digestive gland and, as such, digestive gland is a better indicator of dietary short-time shifts (Stowasser et al. 2006). The isotopic differences can be linked to the protein metabolism and the specific retention of the heavier isotope in each tissue. Higher nitrogen isotope ratios in mantle tissue can reflect higher retention of the heavier isotope in the mantle due to differences in amino acid deposition between tissues (Hobson and Clark 1992).

Lipid content interfered with the isotopic values. The effect of delipidification on the $\delta^{15}N$ and $\delta^{13}C$ isotopic signatures of the digestive gland and mantle was significant. The $\delta^{13}C$ is inversely related to the lipid content, i.e. delipidified tissues were enriched compared to non-delipidified tissues, as already demonstrated by several studies (Tieszen et al. 1983a). $\delta^{13}C$ isotopic signatures clearly distinguished non-delipidified and delipidified tissues while $\delta^{15}N$ was most distinct between delipidified tissues, though mantle and tentacle were similar in the non-delipidified.

This study shows the importance of delipidification of tissues for isotopic composition of $\delta^{15}N$ and $\delta^{13}C$ in cephalopod *O. vulgaris* as demonstrated by (Stowasser et al. 2006) were non removal of lipids prior to isotopic analysis of cephalopod tissues, including the digestive gland, lipid-rich compared to muscle tissues accounted for the inter-tissue variability.

The similarity in isotopic composition between muscle tissues, mantle and tentacle, of this cephalopod, exempts the analysis of both tissues. The low variability in the isotopic values of the studied tissues at the intraespecific spatial level requires a more detailed study on the spatial effect, with greater distances involved. Isotopic values help understanding trophic pathways and food source but the tissues responses are also of great importance for correct conclusions on the investigation of diet variability and environmental changes.

5 Diet-shift of *Octopus vulgaris* in the Algarve: an intra and inter-tissue approach

5.1 Abstract

Marine food web dynamics are nowadays defined with biochemical techniques in addition to the traditional stomach content analysis. Determination of tissue fatty acid content and stable isotope analysis has become common tools to evaluate trophic relationships. The application of the techniques on multiple tissues will give information at both the temporal and spatial scale, aspects that will not be less informed when undertaking single tissue sampling. Variability of food sources at the spatial scale is given by intra-tissue comparison and at temporal scale by inter-tissue comparison.This study on shifts in the diet of the common octopus (*Octopus vulgaris*) from the northeast Atlantic combined the determination of the stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) with fatty acids trophic markers in the digestive gland and mantle tissue. The mantle muscle has a low turnover rate and represents an integrated diet history from a longer period, when compared to the digestive gland, which has a higher turnover rate and represents a more recent diet. The biochemical analyses on tissues of thirty individuals showed more depleted and variable isotopic values for digestive gland and more enriched and less variable values for mantle muscle. Both tissues showed high concentrations of the polyunsaturated fatty acids, DHA (22:6(n-3)) and EPA (20:5(n-3)), tracers of dinoflagellates and diatoms, respectively. Digestive gland had higher concentrations of the monounsaturated fatty acid (MUFA) 18:1(n-9), a tracer for salt marsh plants, marine algae and crustaceans, when compared to the mantle. Concentrations of the fatty acids linolenic acid 18:3(n-3) and linoleic acid 18:2(n-6) showed that there was a limited contribution of terrestrial organic matter to the food web of *O. vulgaris*. There was evidence of a recent dietary shift to food sources depleted in $\delta^{13}C$, with lower concentrations in DHA and EPA, essentially of marine origin and a strong contribution by dinoflagellates to the base of the food web. The main conclusion was that the combination of these

biochemical techniques in multiple tissues was very effective in the study of the trophic dynamics of this traditionally defined opportunistic predator.

5.2 Introduction

The common octopus, *Octopus vulgaris*, is a benthic species common in rocky and sandy subtracts, above 200 m depth. This species has a worldwide distribution (Quetglas et al. 2000, Moreno et al. 2014, Xavier et al. 2015) and an increasing importance as a target species in fisheries and aquaculture (Prato et al. 2011). As such, there is a need to achieve the most complete information on its trophic dynamics (Pinnegar and Polunin 1999, Garrido et al. 2008, Kelly and Scheibling 2012).

O. vulgaris is a short lived animal (12-15 months) with the young settling 1-2 months after hatching and reaching the sub-adult stage at 9-10 months of life (Katsanevakis and Verriopoulos 2006). This cephalopod is a simultaneous terminal once spawner (Rocha et al. 2001) and in the Portuguese coast the spawning season of the population peaks once or twice a year (Lourenço et al. 2012).

Cephalopods have a major ecological role in the structuring and functioning of communities as opportunistic predators of a broad range of benthic and nektonic species but are also preyed upon by fish, birds and mammals (Stowasser et al. 2006, Xavier et al. 2015). Crustaceans are the most frequent prey of *O. vulgaris,* either as planktonic paralarvae or after settlement, followed by molluscs (mainly bivalves), fish and other invertebrates (Smith 2003, García García and Cerezo Valverde 2006, Anderson et al. 2008, Roura et al. 2012, Robin et al. 2014).

O. vulgaris is an important vector for the transfer of energy between components of the trophic web and also between habitats, e.g. river plume to marine benthic food

webs (Phillips et al. 2001, Moreno et al. 2014). River plumes provide good feeding and survival conditions for the younger stages of *O. vulgaris.* Energy transfer along a food chain that includes *O. vulgaris* is continually affected by environmental conditions (Moreno et al. 2014).

Fatty acid analysis (FATM) and stable isotope analysis have become common tools for the study of trophic relationships. The use of multiple tissues give more information at the temporal and spatial scale of ashift in diet, from each animal, undetectable when sampling only one tissue (Alfaro 2006, Budge et al. 2006, Petursdottir et al. 2008, Kelly and Scheibling 2012).

Fatty acid trophic markers (FATM) are fatty acids typically metabolized by certain species and mark their consumption throughout the food web (Phillips et al. 2001) if weak metabolic processes occur in the components of the trophic web (Dalsgaard et al. 2003). Examples of sources of FATM are: salt marsh plants and green algae for oleic acid (*cis*-9-octadecenoic acid; 18:1(n-9)); terrestrial vascular plants for linoleic acid (*cis*-9,12-octadecadienoic acid; 18:2(n-6)) and α-linolenic acid (*cis*-9,12,15-octadecatrienoic acid; 18:3(n-3)); herbivorous copepods for gondoic acid (*cis*-11-eicosenoic acid; 20:1(n-9)), cetoleic acid (*cis*-11-docosenoic acid; 22:1(n-11)) and erucic acid (*cis*-13-docosenoic acid; 22:1(n-13)), with the combination of 20:1(n-9)+22:1(n-9/11) as trophic marker on copepod feeding; diatoms for palmitoleic acid (*cis*-9-hexadecenoic acid; 16:1(n-7)) (Kelly and Scheibling 2012).

The fatty acid composition of herbivores and their consumers reflect the dominant communities of the phytoplankton and the base of the food web. If proportions of EPA (*cis*-5,8,11,14,17-eicosapentaenoic acid; 20:5(n-3)) are higher than DHA (*cis*-4,7,10,13,16,19-docosahexaenoic acid; 22:6(n-3)) then the food web is based on diatoms; if there is a higher proportion of DHA, the food web is based on dinoflagellates. The DHA in consumers is also a fatty acid trophic marker, typical of carnivorous behavior (Dalsgaard et al. 2003, Kelly and Scheibling 2012).

Stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) indicate primary energy sources and relative trophic position, respectively (McCutchan et al. 2003). Stable

isotope ratios change in time towards the isotopic composition of the diet (DeNiro and Epstein 1977, 1978, 1981, Minagawa and Wada 1984, Pinnegar and Polunin 1999, Stowasser et al. 2006). From the diet to consumer tissue, with each trophic step, $\delta^{13}C$ increases by ca.1‰ (Darnaude 2005, De Lange and Van den Brink 2006). $\delta^{15}N$ exhibits a high variation (Adams and Sterner 2000) but, based on classical feeding studies, the accepted $\delta^{15}N$ enrichment with each trophic step is 3-4‰ (Minagawa and Wada 1984, Peterson and Fry 1987).

The real tissue to diet discrimination differences are obtained by controlled feeding experiments as performed by Stowasser et al. (2006) and Redmond et al. (2010). Stable isotopic ratios can be affected by time of year, sex, geographic location (DeNiro and Epstein 1978, 1981, MacNeil et al. 2005, Stowasser et al. 2006) and physiology (Dalsgaard et al. 2003, Lourenço et al. 2014).

$\delta^{13}C$ distinguishes between planktonic and benthic food webs. Values lower than 20‰ are indicative of a pelagic food web, where consumers derive their carbon from phytoplankton. Values enriched above -20‰, are considered typical of a benthic food web (France 1995).

The turnover rate of tissues depend on the time taken by each type of tissue for the incorporation of trophic markers from the assimilated food (Tieszen et al. 1983, Pinnegar and Polunin 1999, MacNeil et al. 2005, Stowasser et al. 2006, Cherel et al. 2009, Del Rio et al. 2009(Cherel et al. 2009a)). Digestive gland tissues have a rapid turnover and will reflect a more recent diet, especially when compared to the mantle which integrates over a longer period of food intake (Stowasser et al. 2006, García-Garrido et al. 2010). The digestive gland is an organ for energy storage (Boucaud-Camou and Boucher-Rodoni 1983, Semmens and Moltschaniwskyj 2000), digestive and enzyme synthesis, nutrient absorption, excretion and detoxification (Boucaud-Camou and Boucher-Rodoni 1983) where dietary fatty acids experience little modification following ingestion (Phillips et al. 2001, Stowasser et al. 2006, García-Garrido et al. 2010).

The primary functions of the mantle are mechanical ventilation, jet propulsion and locomotion (Kier and Thompson 2003) and secondarily, as an energy store (Semmens and Moltschaniwskyj 2000, Kier and Thompson 2003). Specific fatty acids are retained in the mantle for their structural role (e.g. Phillips et al. 2001, Stowasser et al. 2006, García-Garrido et al. 2010).

The aim of this study was to detect dietary shifts, both temporal and spatial, of *O. vulgaris* in the natural environment using stable isotopes and fatty acid trophic markers in tissues with different turnover rates.

5.3 Materials and methods

5.3.1 Study site, sample collection and processing

Octopuses were caught by commercial fishing boats operating in the northeast Atlantic, more specifically, along the southeastern coast of Portugal in May 2013 (Figure 5.3.1).

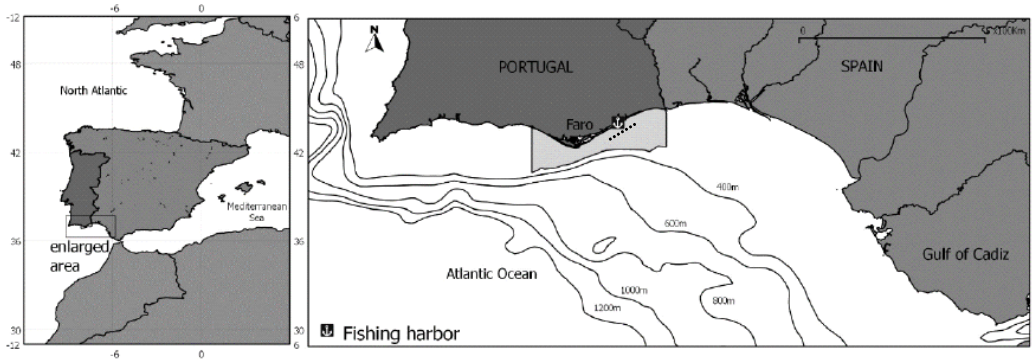

Figure 5.3.1 Map of the fishing harbor in Tavira and the replicate fishing trajectory (dots),parallel to the coastline, adapted from Sonderblohm (2015).

This fishery *métier* is a main-line with thousands of baited traps and it was set along several kilometers at 50-100 m depth offshore of Tavira (37º06'N, 7º31'W). Seven

replicates were collected in a parallel trajectory to the coastline, 1-6 nm from the shore. Replicate sampling was composed of three or five animals from sequential traps attached to a long line connected to the main line.

Samples weighed between 0.75 kg (legal minimum landing weight) and 1 kg, which corresponds to the sub-adult phase of the octopus (Sonderblohm 2015).

A total of thirty animals were kept in ice after the fishery. The mantle and digestive gland were dissected from the animals within 12 hours of sampling. The dissected tissues were immediately frozen at -20°C. This minimized any opportunity for lipid oxidation (Budge et al. 2006) or change in isotopic ratio from the procedures (Stallings et al. 2015).

5.3.2 Sample analyses

5.3.2.1 *Fatty acids analyses*

Lipids were isolated and purified from the tissues, using a simplified version of the method as described by Folch et al. (1957). The lipids were converted into Fatty Acid Methyl Esters (FAMEs) by transesterification. The FAME composition of the extracts was determined by gas chromatography with flame ionization detection (GC-FID) (Webster et al. 2006).

A total of twenty nine FAMEs were identified in the samples. The relative abundance of Fatty Acid Trophic Markers (FATM) indicated the input of organic sources.

5.3.2.2 Stable isotopes analyses, $\delta^{15}N$ and $\delta^{13}C$

Stable isotope analysis was performed on delipidified tissues (DeNiro and Epstein 1977, Tieszen et al. 1983b, Nyssen et al. 2005). Samples were dried to a constant weight at 60°C, powdered using a mortar and pestle and homogenized. Approximately 0.6 mg was loaded into 6x4 mm tin capsules (SerCon).

Samples were combusted and stable isotopes were analyzed in a SerCon Carbon and Nitrogen Isotope Analyser coupled with an element analyser (IRMS, isotope ratio mass spectrometer). The results are reported using the δ notation, in *per* thousand (‰) deviations from international standard reference materials and the analytical precision was <0.4‰ for $\delta^{15}N$ and <0.2‰ for $\delta^{13}C$.

The $\delta^{15}N$ values are in *per mil* deviations from atmospheric nitrogen and calculated as:

$$\delta^{15}N = \left[\left(\frac{\left({}^{15}N/{}^{14}N\right)_{sample}}{\left({}^{15}N/{}^{14}N\right)_{atmN}}\right) - 1\right] \times 10^3$$

The $\delta^{13}C$ values are in *per mil* deviations relative to Vienna Pee Dee Belemnite and calculated as:

$$\delta^{13}C = \left[\left(\frac{\left({}^{13}C/{}^{12}C\right)_{sample}}{\left({}^{13}C/{}^{12}C\right)_{VPDB}}\right) - 1\right] \times 10^3$$

5.3.2.3 Statistical analysis

Statistical analyses from the identified fatty acids, FATM and stable isotopic composition were performed using the program R (R Core Team 2013). Fatty acids not classified as trophic markers and with proportions below 5% were excluded from the analysis.

Data transformation was not applied to avoid giving artificial weight to fatty acids that contributed a small percentage to the total composition (Nyssen et al. 2005).

Inter-tissue variability in FATM was tested with nested ANOVA to analyze differences between mantle tissue and digestive gland within each octopus. Intra-tissue variability was tested with nested ANOVA to check for the replicate effect within tissues and verify spatial variability at the scale of the sampling distance.

The stable isotope values were assessed using a pairwise t-test. This was used to investigate tissue-related differences. ANOVA tested, for each tissue, the effect of distances on the isotopic signatures through replicate comparisons. Normality of the data was tested with Levene test and homogeneity of the variances with the distribution of the residuals.

5.4 Results

5.4.1 Fatty acid signatures of tissues from O. vulgaris

A total of 29 individual fatty acids or fatty acid groupings (only 22:1) were included in the analysis with the average percentage composition ranging from 0.01± 0.01% (16:3) to 28.67±2.02% (22:6(n-3)) for the mantle and from 0.12± 0.09% (16:3) to 24.13±2.93% (22:6(n-3)) for the digestive gland. In *O. vulgaris,* digestive gland and mantle had similar fatty acids trophic markers.

The fatty acid trophic markers that contributed to more than 55% of the normalized area percent in the mantle were: 22:6(n-3), 20:5(n-3) and 20:4(n-6); and in the digestive gland were: 22:6(n-3), 20:5(n-3), 18:1(n-9), 20:4(n-6), 18:1(n-7) and 16:1(n-7).

Both tissues contained a high percentage of polyunsaturated fatty acids (PUFA) with the average across the 30 samples being more than 60% in the mantle and greater than 50% in the digestive gland. For saturated fatty acids (SFA), they comprised an

average of more than 25% in both tissues. In contrast, the average proportions for the monounsaturated fatty acids (MUFA) were generally higher in the digestive gland, the exception being 20:1(n-9) (Table 5.4.1). The digestive gland had greater proportions of palmitoleic acid 16:1(n-7) (3.43%±1.18%); oleic acid, 18:1(n-9) (7.43%±3.57%) and vaccenic acid 18:1(n-7) (3.50%±1.27%) relative to the mantle (0.75%±0.13%; 2.32% ±0.34% and 1.92%±0.24%, respectively).

Herbivorous copepod biomarkers with higher proportions were: 20:1(n-9) in the mantle; and 22:1(n-9/11) in the digestive gland. The trophic marker 20:1(n-9)+22:1(n-9/11) was thus very similar between tissues. Levels of Arachidonic acid 20:4(n-6) were around 5%, in both tissues.

Tissue related differences in fatty acid composition were evident between mantle and digestive gland tissue of *O. vulgaris* but not within tissues. Digestive gland clearly demonstrated a higher variation in the composition of individual fatty acids and FATM. Proportions differed significantly ($p<0.0001$) between tissues within octopus, except for Arachidonic acid 20:4(n-6) and EPA/DHA ($p<0.05$).

When making an overall comparison of the two tissues, the proportions of FATM differed significantly at $p<0.0001$, except for EPA/DHA and PUFA/SFA while the tissue differences were not significant for ARA and 20:1(n-9)+22:1(n-9/11). The tissue replicates, which provide information on the potential for spatial variability, were not significantly different for FATM, except PUFA/SFA ($p<0.05$) (Figure 5.4.1).

Table 5.4.1. Mean fatty acid signatures and ratio ± 1SD in mantle tissue and digestive gland of *O. vulgaris*. Data are expressed as normalized percentages. Components analyzed without uncertainty are asterisked.

Fatty Acids including **Fatty acid Trophic Markers**	Mantle n=30	Digestive Gland n=30
	Mean SD	Mean SD
14:0	1.15 ± 0.29	3.03 ± 1.04
14:1(n-5)	0.05 ± 0.02	0.21 ± 0.17
15:0	0.16 ± 0.21	0.36 ± 0.48
16:0	19.06 ± 1.13	13.95 ± 1.11
16:1(n-7)	0.75 ± 0.13	3.43 ± 1.18
16:2*	0.06 ± 0.04	0.68 ± 0.29
16:3*	0.01 ± 0.01	0.12 ± 0.09
16:4*	0.03 ± 0.05	0.20 ± 0.13
17:0	1.17 ± 0.37	1.18 ± 0.34
18:0	7.51 ± 2.22	8.13 ± 2.46
18:1(n-9)	2.32 ± 0.34	7.43 ± 3.57
18:1(n-7)	1.92 ± 0.24	3.50 ± 1.27
18:2(n-6)	0.41 ± 0.10	0.99 ± 0.26
18:3(n-6)	0.08 ± 0.05	0.18 ± 0.08
18:3(n-3)	0.09 ± 0.03	0.67 ± 0.22
18:4(n-3)	0.12 ± 0.05	0.97 ± 0.43
20:0	0.11 ± 0.14	0.40 ± 0.13
20:1(n-11)	0.33 ± 0.38	0.83 ± 0.50
20:1(n-9)	3.04 ± 0.32	1.83 ± 0.58
20:2(n-6)	0.85 ± 1.39	0.84 ± 0.28
20:3(n-3)	0.26 ± 0.07	0.27 ± 0.10
20:4(n-6)	5.42 ± 1.17	4.73 ± 1.99
20:4(n-3)	0.21 ± 0.12	0.63 ± 0.18
20:5(n-3)	21.97 ± 1.44	15.98 ± 3.16
22:1(n-9/11)	0.13 ± 0.26	1.05 ± 1.24
21:5(n-3)	0.66 ± 0.45	0.83 ± 0.60
22:5(n-3)	3.09 ± 2.65	2.70 ± 1.04
22:6(n-3)	28.67 ± 2.02	24.13 ± 2.93
24:1(n-9)	0.32 ± 0.23	0.67 ± 0.23
SFA	29.17 ± 2.46	27.12 ± 2.41
MUFA	8.87 ± 0.91	18.95 ± 4.38
PUFA	61.86 ± 2.84	53.93 ± 4.06
PUFA/SFA	2.15 ± 0.33	2.01 ± 0.27
16:1(n-7)/16:0	0.04 ± 0.01	0.25 ± 0.09
18:1(n-9)/18:1(n-7)	1.22 ± 0.19	2.28 ± 1.13
18:2(n-6)+18:3(n-3)	0.51 ± 0.10	1.67 ± 0.46
20:1(n-9)+22:1(n-9/11)	3.18 ± 0.42	2.88 ± 1.50
20:5(n-3)/22:6(n-3)	0.77 ± 0.07	0.68 ± 0.21
22:6(n-3)/20:5(n-3)	1.31 ± 0.13	1.58 ± 0.43
∑C16	19.92 ± 1.21	18.39 ± 1.73
∑C16/∑C18	1.71 ± 0.66	0.89 ± 0.17
∑C18	12.46 ± 2.29	21.20 ± 3.00

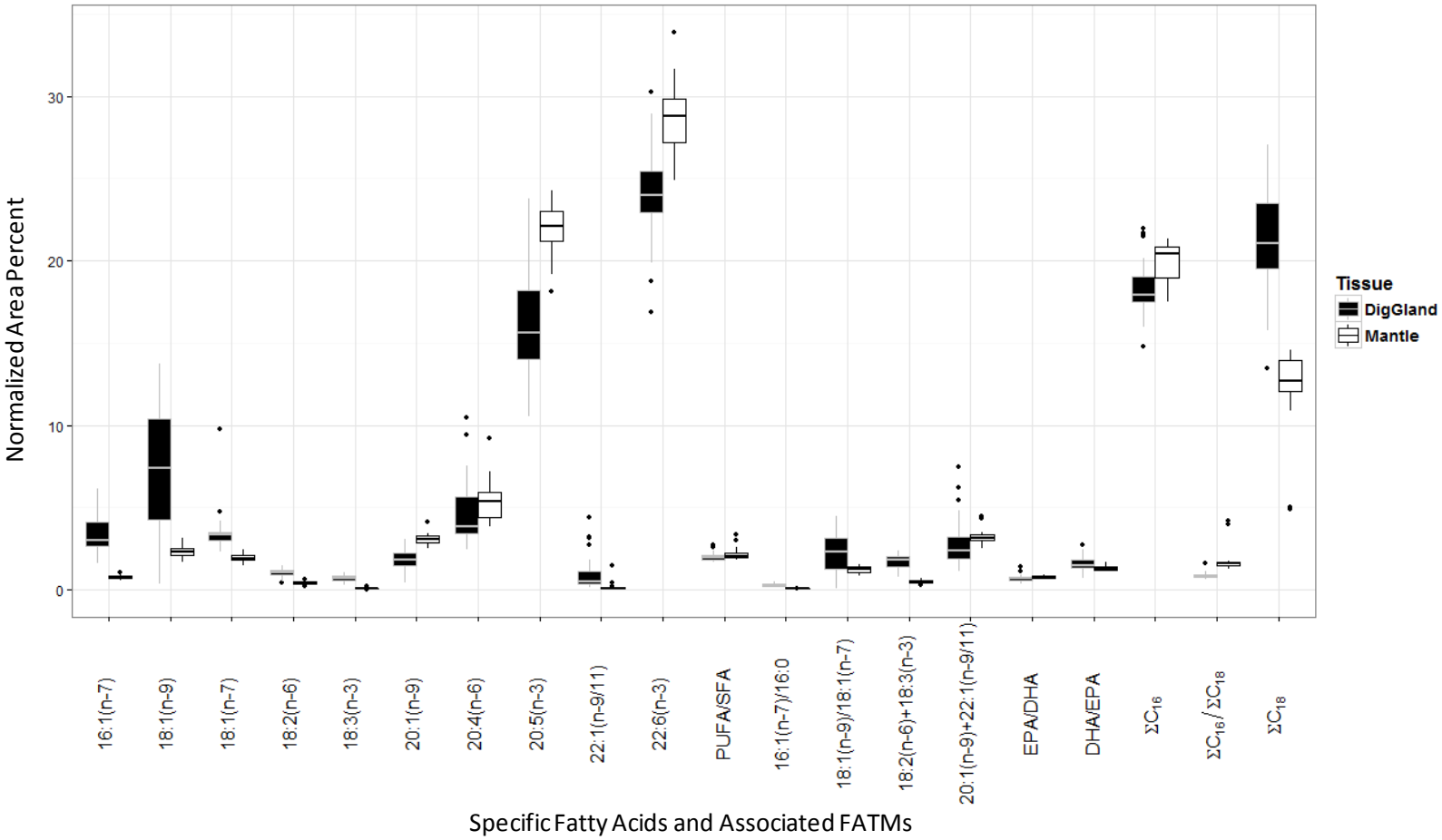

Figure 5.4.1 Boxplots of the normalized area percent of the specific fatty acids and associated fatty acid trophic markers for mantle tissue (white) and digestive gland (black) of *O. vulgaris*.

Isotopic signature of* O. vulgaris *tissues

$\delta^{13}C$ and $\delta^{15}N$ values showed tissue related differences (Figure 5.4.2). $\delta^{13}C$ mean values of the digestive gland (-18.46‰ ± 1.53‰) were significantly more depleted ($p<0.0001$), and showed a larger standard deviation, relative to the mantle (-15.89‰ ± 0.74‰). $\delta^{15}N$ mean values of the digestive gland (8.34‰ ± 0.67‰) were significantly more depleted ($p<0.0001$) relative to the mantle tissue (10.65‰ ± 0.71‰).

Between replicates, there was a marginally significant spatial effect for the difference in the $\delta^{15}N$ within tissues ($p<0.05$) whereas for $\delta^{13}C$ values, there was no evidence of any spatial effect.

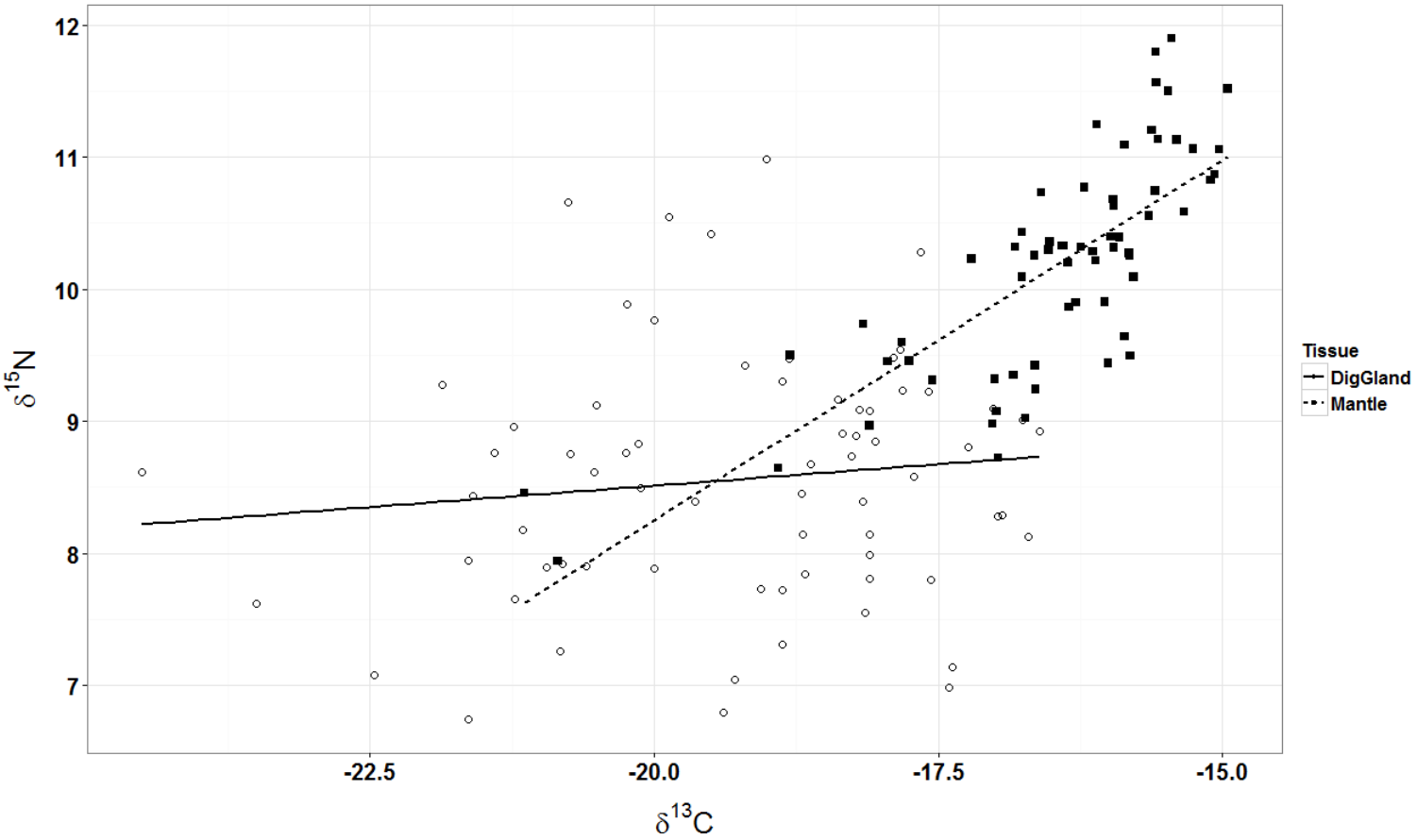

Figure 5.4.2 $\delta^{15}N$ versus $\delta^{13}C$ for digestive gland and mantle tissues of *O. vulgaris*. The black line represents the digestive gland isotopic values smoothed line and shows a relatively small increase in the $\delta^{15}N$ with an increase in the $\delta^{13}C$ isotopic values (open circles). The dotted line represents mantle isotopic values smoothed line. This emphasizes that there is an high increase in the isotopic values of $\delta^{15}N$ with an increase in the isotopic values of $\delta^{13}C$ (black squares). Each data point is an individual analysis.

5.5 Discussion

5.5.1 Energy sources in Octopus vulgaris trophic web

Stowasser et al. (2006) observed during controlled feeding of cephalopods, tissue specific differences in isotopic values and fatty acids, suggesting these were related to metabolic differences between the tissues. Metabolic turnover rates of dietary protein and dietary lipid have an effect on the stable isotope and fatty acid composition in each tissue reflecting the food items assimilated at different periods of feeding (MacNeil et al. 2005, Redmond et al. 2010). The more metabolically active tissues have faster turnover rates than less metabolically active tissues (Tieszen et al. 1983b). In controlled experiments and for stable isotope modeling, individuals are at an equilibrium with their diet, but in the natural environment complementary biochemical analyses and stomach content analysis need to confirm dietary composition (MacNeil et al. 2005, Stowasser et al. 2006).

In the southern Portugal, the cephalopod *Octopus vulgaris* has a diet composed of a broad range of species (Smith 2003, Xavier et al. 2015) this being influenced by temporal patterns (Moreno et al. 2014). Differences in stable isotopes and the fatty acids used as trophic markers have not been used to study temporal and spatial diet shifts for octopus under natural conditions. Given that two functionally and metabolically distinct tissues, digestive gland and mantle muscle, reflect diet at different periods, it was possible to detect inconsistencies in the diet with time at the spatial scale of the study and the recent shift of primary producers at the base of the food web (Kurle and Worthy 2002).

The enriched $\delta^{13}C$ values in tissues showed marine phytoplankton, microphytobenthos or seagrasses are the base of the food web (Connolly et al. 2009, Bouillon et al. 2011) which contrasts with estuarine phytoplankton, depleted and highly variable in $\delta^{13}C$ values (De Lange and Van den Brink 2006, Kharlamenko et al. 2008, Bouillon et al. 2011). Octopuses change their feeding to benthic prey (e.g. crabs and bivalves) when

they settle in the benthos (Smith 2003, García García and Cerezo Valverde 2006, Roura et al. 2012). These prey are closely associated with the benthic productivity (Piché et al. 2010). Thus, in the most recent feeding period, microphytobenthos was the most potential contributor for the base of the food web, with $\delta^{13}C$ values that range from -23‰ to -12‰, e.g. (Bouillon et al. 2011). Nevertheless, the dominance of the dinoflagellates trophic marker DHA (22:6(n-3)) in the tissues tends to substantiate the fact that the octopus food web was dinoflagellate-based (Dalsgaard et al. 2003).

Dinoflagellates are a primary producer dominant in the waters of southern Portugal due to weak upwelling events and low freshwater inputs, both of which tend not to favor diatom blooms (Garrido et al. 2008, Moreno et al. 2014). The diet being of marine origin for the octopus in this area (Stowasser et al. 2006) was further supported by the high proportions of EPA (20:5(n-3)) ((Prato et al. 2010, Biandolino et al. 2010, Estefanell et al. 2012b).

Previously Rosa et al. (2002) showed *O. vulgaris* from the south coast of Portugal fed most intensely on bivalves. However, this present study does not support bivalves as the main food item of *O. vulgaris* since bivalves have higher proportions of palmitoleic acid, 16:1(n-7) and lower proportions of arachidonic acid (ARA), EPA and DHA (Biandolino et al. 2010) and these proportions are not reflected in the tissues of *O. vulgaris* obtained in this study.

5.5.2 Temporal and spatial diet shifts in Octopus vulgaris

Mantle muscle and digestive gland have different functional roles and physiologies and distinct, tissue-related fatty acid compositions were anticipated. Octopuses are recognized as organisms containing high levels of DHA (22:6(n-3)) (Piché et al. 2010), palmitic acid (16:0) and EPA (20:5(n-3)) (Navarro and Villanueva 2003, Stowasser et al. 2006, Miliou et al. 2006, García et al. 2011, Estefanell et al. 2012b). The proportions of the trophic markers 18:2(n-6) and 18:3(n-3), both of which reflect the input of terrestrial matter in the food web (Budge and Parrish 1998, Pernet et al. 2012b), showed that this source was irrelevant for O. vulgaris during the period of relevance

for the digestive gland and mantle. The most likely justification for the low contribution of terrestrial sources is the low river runoff from the main river - Guadiana River – in southern Portugal (Moreno et al. 2014), during the year preceding the study (http://snirh.pt).

$\delta^{13}C$ values took 3-10 days in digestive gland and 10 days in the mantle to reflect the isotopic values of the diet for the squid *Lolliguncula brevis* (Stowasser et al. (2006). Navarro and Villanueva (2003) also showed a trophic marker memory of 10 days for *O. vulgaris* fatty acids when changed to a new diet. Any change in dietary contents is reflected quicker in the digestive gland (less than 10 days of diet) and slower in the mantle (more than 10 days). Changes in the copepod biomarkers, 20:1(n-9) and 22:1(n-9/11) attributable to environmental conditions that affected the base of the food web were within the time integration periods described by both tissues.

The differences between tissues in respect of oleic acid (18:1(n-9)) reveal a possible increase in the predation of crustaceans (Prato et al. 2010, Estefanell et al. 2012b) or herbivores, since marine algae or seagrasses have high levels of oleic acid (Bouillon et al. 2011). Besides dietary input, high proportions of oleic acid are tissue specific and dependent on physiological requirements (Mayor et al. 2013). Low values of 18:1(n-9) were reported in the mantle tissues of *O. vulgaris* by Miliou et al. (2006) and Prato et al. (2011) and high values in the digestive gland were reported by Mayor et al. (2013) and Lourenço et al. (2014) due to the life cycle dependency on 18:1(n-9). The proportion of 18:1(n-9) in digestive gland is not similar across studies and this was clearly demonstrated by Lourenço et al. (2014). Further, the proportions of 18:1(n-9) are affected by the conversion to 20:1(n-9) (Dalsgaard et al. 2003). Oleic acid must be combined with other trophic markers due to its physiological and metabolic role.

Diatoms and dinoflagellates are the dominant phytoplankton groups and therefore the most important energy source for zooplankton. The differences in the biochemical composition of phytoplankton will be marked in the trophic transfers to higher trophic levels (Wasmund et al. 2017). Diatoms have EPA as the major fatty acid in their composition while dinoflagellates have DHA (Dalsgaard et al. 2003). Dinoflagellates bloom when the winter-spring diatom bloom goes through a period of decreased

growth (Smayda and Trainer 2010) and via the trophic web, this dominance can be perceived at higher trophic levels. In the tissues of the octopus, relatively lower levels of EPA, when compared to DHA, were evident and thus indicate the dominance of the dinoflagellate group at the base of the food web (Dalsgaard et al. 2003, Kelly and Scheibling 2012).

The elevated levels of DHA and EPA in the *O. vulgaris* tissues correlates with the food items in the typical diet of this consumer and specific physiological requirements of the mantle or neural tissues (Sargent et al. 1995, Mayor et al. 2013). The strong dependency of these fatty acids on the physiological requirements was confirmed by García et al. (2011) and Prato et al. (2011) who reported high similarities in the DHA to EPA ratio in tissues of this species, not related to dietary contents, (≈ 1.5). As such, these fatty acids combined with other markers will provide more accurate information on trophic interactions. The digestive gland, which represents the recent feeding period, was reduced in EPA (16.0±3.2%) and DHA (24.1±2.9%), relatively to the mantle which covers a longer feeding period. In contrast, 18:1(n-9) was greatly increased in the more recent diet. This clearly indicates a recent modification of the base of the food web or in the trophic dynamics.

In controlled feeding experiments of squid, tissue related differences in stable isotope values were related to metabolic turnover of each tissue and different tissues represent periods of feeding that increase with decreasing metabolic turnover Stowasser et al. (2006). Similar isotopic values between tissues indicate similar dietary inputs over different feeding periods, i.e. feeding specialization in a community with constant environmental conditions.

The results of this study imply lack of consistency in the diet of the octopuses during the periods of time represented by the tissues analyzed. This situation is quite well accepted due to the wide range of seasonal dietary choices that inhibit steady state diet conditions since *O. vulgaris* is a generalist predator in the wild and consumer of prey of different trophic levels. The $\delta^{15}N$ values show a mixed diet at the temporal scale with the longer feeding period resulting in a more enriched $\delta^{15}N$ food sources,

like crustaceans (Rosa et al. 2002, Stowasser et al. 2006) or with prey connected to organic matter enriched in $\delta^{15}N$ (Darnaude 2005).

The $\delta^{15}N$ values could indicate variability in trophic level of octopus only if $\delta^{15}N$ values of the primary producers were available (Post 2002). For steady isotopic values of the primary producer, $\delta^{15}N$ values in the recent feeding period imply a decrease in the trophic level of this consumer due to a reduction in the number of trophic steps otherwise $\delta^{15}N$ values of energetic sources decreased in time.

The $\delta^{13}C$ values in the digestive gland were depleted in $\delta^{13}C$ values relative to the mantle. This variance between tissues is not explained by the lipid content in the digestive gland (Pinnegar and Polunin 1999) since all tissues were delipidified. These differences in terms of dietary analysis reflect a decrease in the $\delta^{13}C$ values of the prey or the primary producers. Nevertheless, for suitability of this stable isotope in tracking temporal diet, influences of metabolic mechanisms must be acknowledged as well as the composition of the aminoacids in the diet (Chikaraishi et al. 2009, Mayor et al. 2013, McMahon et al. 2015).

The enriched and less variable values of $\delta^{13}C$ values for the longer feeding period (as represented by the mantle), comply with the weak influence of river flow and low contribution of estuarine matter or terrestrial matter to this marine trophic web. Multiple primary producers from the microphytobenthos (Bouillon et al. 2011) with contribution of inshore carbon (France 1995) at the base of the food web, are thus suggested as the main cause of the enriched values in the mantle whereas in the recent diet there is an apparent increase of more depleted sources. The difference of $\delta^{13}C$ values between tissues, from enriched above -18‰ to below -20‰ strongly showed a shift from a benthic based food web to a phytoplankton based food web.

A greater availability of primary producers at the base of the food web is also an explanation for the high variability of $\delta^{13}C$ values in the digestive gland, as well as herbivores of microphytobenthos or marine phytoplankton and detritivorous (Kharlamenko et al. 2008).

The current study established similarity in diet across the spatial level of sampling. Distances were short and benthic assemblages and environmental conditions likely similar plus the foraging behavior of the octopuses weakened the spatial effect at this small scale.

O. vulgaris is a generalist predator but can be a specializing predator (Anderson et al. 2008) if similar prey were selected at the different sites. Carnivorous prey were a primary choice in the diet of the octopuses, as indicated by the trophic marker indicator 22:6(n-3)/20:5(n-3)>1, if physiological requirements are considered not significant (García et al. 2011, Prato et al. 2011).

5.6 Concluding remarks

This study shows that trophic marker analyses of multiple tissues provide greater resolution in trophic dynamics than the use of a single tissue. Nevertheless, metabolic differences between tissues provide detail with respect to the trophic dynamics of octopus and distinguish from metabolic requirements. The role of octopus as an opportunistic consumer was clearly demonstrated by the relative compositions of the stable isotopes and fatty acids trophic markers. The variability of diet items can better be evaluated if temporal sampling and other techniques as stomach contents analysis are included in the diet analysis.

In conclusion, stable isotopes and fatty acids were useful to identify main trophic pathways and detect differences in diet at the temporal scale in the octopus from the coastal waters of southern Portugal. However, the interpretation of the spatial effect was limited due to the small distances covered by the sampling. Multiple tissue sampling took account of tissue metabolic differences in trophic markers to achieve temporal scales for diet shifts and minimize sampling effort. This tactic detected a recent trophic shift and a non steady-state condition at the base of the food web. The results showed that the base of the trophic food web of this benthic cephalopod was

marine phytoplankton-based with a dominance of dinoflagellates with a recent increase in the contribution of benthic algae.

Nevertheless, additional trophic interactions and baseline knowledge of primary producers and other intermediate components of the food web are necessary to describe the trophic ecology and trophic dynamics of this cephalopod. Studies on the fatty acids biosynthesis and on the isotopic values in the aminoacids from the different tissues in the octopus are required to determine the applicability of these trophic markers to track energy flow and distinguish diet input from physiological requirements.

6 Pelagic bait as food supply to a benthic consumer

6.1 Abstract

Pelagic fish is popular bait in the octopus fisheries. The increase in fishing effort leads to an increase in the bait introduced as food in the trophic web of the octopus. In this study, the importance of this bait is analyzed with trophic markers, stable isotopes and fatty acids for the trophic web of the *Octopus vulgaris* of the southern Algarve. The variability of diet is assessed with the analysis of several tissues of the octopus. This octopus fishery uses traps baited with pelagic fish, e.g. *Scomber* sp. and *Trachurus trachurus* and questions arise for the importance of this bait as chronic food supply for this cephalopod and for a trophic link between the pelagic and the benthic environment. Stomach contents and trophic markers of stomach contents demonstrated the fish bait was the last meal of the octopus caught in the traps. Inter tissue differences (digestive gland, mantle muscle and stomach contents) in the $\delta^{15}N$ and $\delta^{13}C$ values and fatty acid trophic markers of octopus suggested dietary temporal shifts with an adjustable feeding behavior to the available energy sources. Fish bait in the traps was an opportunistic feeding but not a regular food source, contrary to crustaceans, due to the high levels of EPA, 20:5(n-3) in the tissues, typically high in crustaceans. In the octopus, the high levels of EPA, 20:5(n-3) indicated crustaceans as a preferred and available food item and the levels of DHA, 22:6(n-3) indicated a dinoflagellate based food web. The dietary shifts in the last meal, strongly identified by the high levels of 18:1(n-9) in the bait and stomach contents, support the opportunistic feeding behavior of the cephalopod for this unusual food. Overall, the contributions of the pelagic fish as bait to the trophic web of *O. vulgaris* were limited to the last feeding occasion. These pelagic fish are products of low quality and economic value and since these fish result from commercial catches, ecological impact for bait harvesting is not linked with octopus fishery.

6.2 Introduction

The cephalopod *Octopus vulgaris* is one of the most abundant and ecologically important cephalopods in the Atlantic Ocean (Quetglas et al. 2000, Moreno et al. 2014). This species links components in the marine benthic food web (Xavier et al. 2015) for the ability to prey on crustaceans, fish, bivalves and other invertebrates (Giménez and Garcia 2002, Smith 2003, Prato et al. 2010, Roura et al. 2012) and as prey of fish, birds and mammals (Xavier et al. 2015).

O. vulgaris plays a role in the transfer of energy from the pelagic to the benthic environment due to the meroplanktonic life history strategy (Katsanevakis and Verriopoulos 2006) and is a species highly sensitive to environmental variability (Lourenço et al. 2012, Moreno et al. 2014). In the benthic environment, the octopus is known for its high site fidelity and limited migrations, where scarcity of food and shelter are the main causes for movements (Katsanevakis and Verriopoulos 2006, Mereu et al. 2015).

The increasing worth of the octopus in the coastal fisheries comes with the decline in the finfish fisheries (Moreno et al. 2014). Catches rise by 50% in the Portuguese coast since the late 1980's is sustained by the long breeding season and a continuous recruitment (Lourenço et al. 2012, Sonderblohm et al. 2014). In the Algarve, industrial and artisanal fleets sustain the high socio-economical importance of the octopus fishery that represents 40% of the landed fishing biomass. The main fishing technique in the coastal area is the baited trap - a simple and passive gear, highly species selective with minor ecological impact on the benthic environment (FAO 2005, Taborda 2012, Pita et al. 2015, Emery et al. 2016). The bait is set inside the trap to attract the cephalopod that seeks refuge for hiding and breeding and once trapped, the cephalopod hardly escapes (Guerra et al. 2015, Mereu et al. 2015).

Dead pelagic fish is common bait due to its availability, low cost, easy storage, on-board handling and luring effect by chemical cues; against live crabs that are used as

bait for more soaking time of the trap (Archdale and Kawamura 2011). The increase in fishing effort enlarges the input into a relatively small area of bait biomass that was produced in a much bigger area (Saila et al. 2002). Bait produced from offshore pelagic fish will thus, connect spatially separated and dissimilar food webs (Connolly et al. 2005b, 2009, K. Abrantes et al. 2015).

The octopus is widely known as an opportunist feeder. Stomach contents show the last feeding opportunities and longer feeding habits are detected by trophic markers, as fatty acids and stable isotopes. These show the temporal variability of trophic sources and energetic connectivity to other environments. Within and among tissues, differences in trophic markers reveal dietary shifts, e.g. digestive gland reflects recent period of feeding while the mantle integrates a longer period of feeding (MacNeil et al. 2005, Stowasser et al. 2006).

Examples of markers and the primary producers in the food webs are: EPA 20:5(n-3) (diatoms); palmitoleic acid 16:1(n-7) (diatoms); linoleic acid 18:2(n-6) (vascular plants) and γ- linolenic acid 18:3(n-3) (vascular plants and salt marsh plants); asclepic acid 18:1(n-7) (green algae); DHA 22:6(n-3) (dinoflagellates). The ratio 22:6(n-3) to 20:5(n-3) indicates the dinoflagellate dominance over diatoms in the phytoplankton that sustains the food web and a carnivory feeding mode (Dalsgaard et al. 2003, MacNeil et al. 2005, Stowasser et al. 2006, Bouillon et al. 2011).

This study is a preliminary assessment of the trophic connectivity between different environments through the supply of pelagic bait to a benthic food web.

6.3 Materials and methods

6.3.1 Fishing ground and sampling

Octopus vulgaris specimens were collected from catches of commercial fisheries from the fishing vessels under the command of Mestre José Maria and Mestre Isaltino in May 2013. The fishing *métier* was trap baited with thawed pelagic fish: *Scomber* sp. and *Trachurus trachurus*. The fishing gear was set along a main line disposed for several kilometers parallel to the coast in the eastern Algarve coast between 37º 06'N and 7º 31'W at depths of 50m to 100m depth (Figure 6.3.1).

Three to five cephalopods from sequential traps composed a replicate, each replicate separated by regular intervals of traps. Individuals weighted between 0.75kg (legal minimum landing weight) and 1kg.

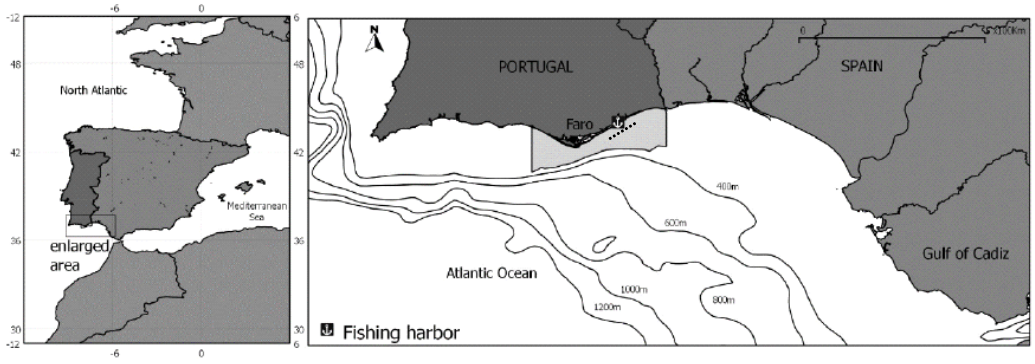

Figure 6.3.1 Map of octopus sampling sites along the coastline in the fishing ground, adapted from Sonderblohm (2015).

6.3.2 Sample analyses

6.3.2.1 Sample processing and sampling

Octopuses were kept on ice and dissected within the day of catch for mantle, digestive gland and stomach contents. Stomach contents were homogenized. Bait was dissected for white muscle: *Scomber* sp. (n=2) and *Trachurus trachurus* (n=1). All samples were frozen at -20°C until analysis.

6.3.2.2 Fatty acids analyses

Lipids were extracted from samples (2g) with a modification of the method of Folch et al. (1957) to adjust to the lipid concentration and the sample size. In brief, lipids were extracted using a chloroform-methanol solvent (2:1 v/v) solvent system. The lipid extract was transferred to a vial and converted to fatty acid methyl esters (FAME) by transesterification overnight (incubated at 50°C) using distilled toluene and methanol-containing sulphuric acid (1% v/v). The methyl esters were extracted into *iso*-hexane and dried over anhydrous sodium sulphate at -20°C until analysis. The resulting FAMEs were determined by gas chromatography with flame ionization detection (GC-FID) using an Agilent (Hewlett-Packard) 5890 Series II gas chromatograph and equipped with a cool, on-column auto injector, fitted with a fused silica capillary column (0.25mm i.d. x 30m) coated with a 0.25 µm film of 50% cyanopropyl. The FAMEs were injected (1µL) at 60°C. The oven temperature was ramped at 25°C/min from 60°C to 150°C and then 1°C/min to 200°C. The temperature was held constant for 10 minutes before final elevation at 50°C/min to 230°C where it was held for 5 minutes. The detector was set at 300°C. Nitrogen was used as the carrier gas (1mL/min) (Webster et al. 2006).

Laboratory reference materials and procedural blanks were included in each batch of samples. A total of 29 FAME were identified from the GC retention times of the

laboratory standards. Assurance checks were made for the GC retention times and the esterification process with the analyses of the laboratory reference materials. Gas chromatography-mass spectrometry (GC-MS) analysis of fish samples confirmed the constituents. Data were expressed as normalized area percentage of the identified fatty acids.

The typical Fatty Acid Trophic Markers (FATM) used in this study follows the description of Dalsgaard et al. (2003), Naczk et al. (2004) and Kelly and Scheibling (2012).

6.3.2.3 Stable isotopes analyses

Delipidified tissues from the fatty acids extraction were used for the stable isotopes analysis. This delipidification will prevent the effect of lipid contents on isotopic composition (DeNiro and Epstein 1977, Tieszen et al. 1983a, Nyssen et al. 2005).

Delipidified tissues were dried at 60°C for a minimum of 24h, until constant weight, then ground to a homogeneous powder with a mortar and pestle. Approximately 0.6mg of each sample was sealed in a SerCon tin capsule 6x4 mm. Samples were analyzed for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ with a SerCon Carbon and Nitrogen Isotope Analyser coupled with an element analyser (IRMS, isotope ratio mass spectrometer) in dual isotope mode to measure $\delta^{13}C$ and $\delta^{15}N$ from each capsule. Isotope ratios reported in conventional delta (δ) notation as parts per thousand (‰) are relative to the international standard Vienna Pee Dee Belemnite (C) and atmospheric nitrogen (N) according to the equation:

$$\delta R = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000$$

where $R=^{13}C/^{12}C$ or $R=^{15}N/^{14}N$. Replicate measurements errors of internal laboratory standards were <0.4‰ for $\delta^{15}N$ and <0.2‰ for $\delta^{13}C$.

6.3.3 Statistical analysis

The data of the trophic markers were uploaded into R (R Core Team 2013). No transformation of normalized percentages of fatty acids avoided artificial weighting of trophic markers (Nyssen et al. 2005) and fatty acids not considered as trophic markers were filtered out for values under 5%. The remaining data comprised the data set for the subsequent trophic markers analysis.Differences in fatty acids trophic markers were tested for significance using a two-way analysis of variance with repeated measures for the factor tissue (with three levels: digestive gland, mantle and gut contents) and the factor replicate and the interaction of tissue with replicates.

The effect of the Tissue within each Replicate was tested with nested ANOVA, which parceled out the replicate variability. The data set was analyzed for normality with Levene's test and analyzed for homogeneity of the variances with the distribution of the residuals. A *posteriori* Tukey HSD was performed for tissues considered significantly different ($p<0.05$).

6.4 Results

6.4.1 Fatty acid composition

The analysis was applied to *O. vulgaris* individuals where the fatty acids extraction was successful for the three tissue types (n=16). The main saturated fatty acids (SFA) were palmitic acid, 16:0 (>15%) and stearic acid 18:0 (7%). Oleic acid 18:1(n-9) was the dominant monounsaturated fatty acid (MUFA), mainly in digestive gland tissue and stomach contents. Polyunsaturated fatty acids (PUFA) comprised over 50% of fatty acids content in the mantle and digestive gland of *O. vulgaris* , with DHA 22:6(n-3) at higher concentrations than EPA 20:5(n-3) and ARA 20:4(n-6); other PUFAs were lower than 4%.

Despite the low concentrations of palmitoleic acid 16:1(n-7), linoleic acid 18:2(n-6), γ-linolenic 18:3(n-3) and asclepic acid 18:1(n-7), these trophic markers were further used in analysis. DHA was most important than EPA in stomach contents comparatively to tissues. More than 40% of the fatty acid profile was composed of 16:0 and the trophic marker 22:6(n-3) (Table 6.4.1, Figure 6.4.1).

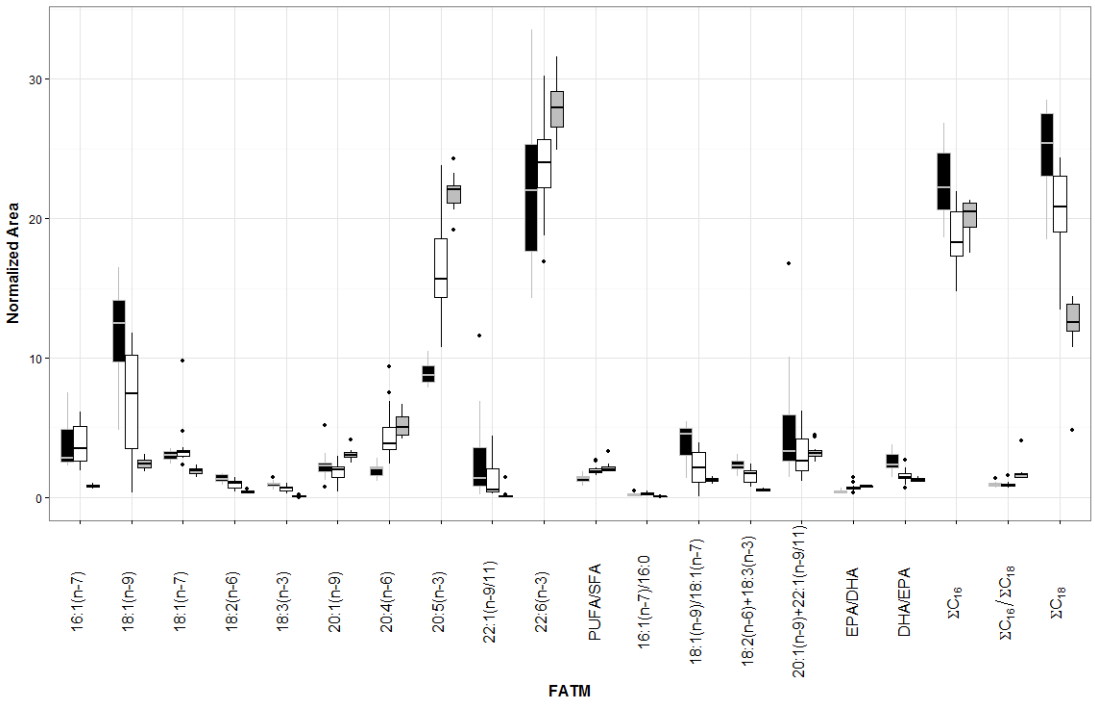

Figure 6.4.1 Boxplots of Fatty Acid Trophic Markers of *Octopus vulgaris* in the Mantle (grey) and Digestive Gland (white) tissues and the Stomach contents (black). Data are expressed as normalized area percentage.

The contents of stomachs among cephalopods varied greatly in the concentrations of DHA and were significantly different for the remaining fatty acid trophic markers in comparison with the mantle but not significantly different for 16:1(n-7); 18:1(n-9); 20:1(n-9); DHA; MUFA; Total C16/ Total C18 in comparison with the digestive gland.

Mantle and digestive gland were not significantly different for ARA; PUFA/SFA; EPA/SFA; DHA/EPA; Total C16 (Table 6.4.1, Figure 6.4.1).

Table 6.4.1 FAME components ±1SD in mantle, digestive gland tissues and stomach contents of *Octopus vulgaris*. Data were expressed as normalized percentages (those without uncertainty calculated are asterisked).

Fatty acids and **Fatty Acid Trophic Markers**	Mantle n=16		Digestive Gland n=16		Gut contents n=16	
	Mean	SD	Mean	SD	Mean	SD
14:0	1.26	± 0.34	3.29	± 1.06	3.93	± 0.84
14:1(n-5)	0.05	± 0.02	0.20	± 0.13	0.13	± 0.07
15:0	0.21	± 0.25	0.36	± 0.39	0.99	± 0.28
16:0	19.21	± 1.07	14.12	± 1.25	17.88	± 1.90
16:1(n-7)	0.80	± 0.13	3.74	± 1.34	3.70	± 1.62
16:2*	0.06	± 0.05	0.55	± 0.27	0.59	± 0.22
16:3*	0.01	± 0.01	0.13	± 0.09	0.16	± 0.08
16:4*	0.05	± 0.05	0.23	± 0.14	0.21	± 0.13
17:0	1.19	± 0.34	1.12	± 0.34	1.00	± 0.46
18:0	7.34	± 2.07	7.79	± 2.86	7.47	± 1.62
18:1(n-9)	2.42	± 0.34	7.00	± 3.79	11.53	± 3.37
18:1(n-7)	1.92	± 0.26	3.65	± 1.71	3.00	± 0.37
18:2(n-6)	0.43	± 0.09	0.96	± 0.29	1.36	± 0.27
18:3(n-6)	0.09	± 0.05	0.20	± 0.10	0.14	± 0.03
18:3(n-3)	0.09	± 0.04	0.65	± 0.21	0.96	± 0.20
18:4(n-3)	0.12	± 0.05	1.02	± 0.43	1.32	± 0.53
20:0	0.13	± 0.17	0.39	± 0.14	0.44	± 0.18
20:1(n-11)	0.47	± 0.48	0.85	± 0.66	0.54	± 0.11
20:1(n-9)	3.09	± 0.35	1.80	± 0.64	2.30	± 1.00
20:2(n-6)	1.15	± 1.88	0.80	± 0.33	0.83	± 0.28
20:3(n-3)	0.27	± 0.07	0.25	± 0.10	0.37	± 0.10
20:4(n-6)	5.19	± 0.81	4.54	± 2.00	1.93	± 0.47
20:4(n-3)	0.24	± 0.16	0.64	± 0.17	0.71	± 0.08
20:5(n-3)	21.85	± 1.18	16.28	± 3.45	8.96	± 0.92
22:1(n-9/11)	0.18	± 0.34	1.33	± 1.33	2.66	± 3.03
21:5(n-3)	0.63	± 0.47	0.64	± 0.43	1.05	± 0.36
22:5(n-3)	3.22	± 2.64	2.85	± 1.03	2.27	± 0.35
22:6(n-3)	28.05	± 2.05	23.88	± 3.56	22.42	± 6.01
24:1(n-9)	0.29	± 0.24	0.71	± 0.27	1.08	± 0.38
SFA	29.34	± 2.65	27.11	± 2.48	31.78	± 3.49
MUFA	9.21	± 1.03	19.27	± 4.77	24.93	± 6.28
PUFA	61.35	± 3.13	52.97	± 4.90	42.33	± 6.90
PUFA/SFA	2.12	± 0.37	1.98	± 0.32	1.35	± 0.30
16:1(n-7)/16:0	0.04	± 0.01	0.27	± 0.10	0.21	± 0.09
18:1(n-9)/18:1(n-7)	1.27	± 0.15	2.20	± 1.24	3.96	± 1.33
18:2(n-6)+18:3(n-3)	0.53	± 0.09	1.61	± 0.48	2.32	± 0.45
20:1(n-9)+22:1(n-9/11)	3.26	± 0.51	3.12	± 1.53	4.97	± 3.92
20:5(n-3)/22:6(n-3)	0.78	± 0.08	0.71	± 0.25	0.43	± 0.12
22:6(n-3)/20:5(n-3)	1.29	± 0.13	1.55	± 0.46	2.52	± 0.68
∑C16	20.13	± 1.15	18.77	± 2.13	22.53	± 2.59
∑C16/∑C18	1.74	± 0.63	0.93	± 0.20	0.92	± 0.16
∑C18	12.32	± 2.28	20.62	± 3.13	24.82	± 3.14

Oleic acid 18:1(n-9) was the trophic marker that made a distinction between the stomach contents and the tissues of the cephalopod and also between bait and mantle tissue of the octopus. Major fatty acids in the bait were EPA and DHA, with DHA values reaching two to three fold the proportions of EPA and always higher than for any tissue of octopus. The EPA proportions in the fatty acid profile were similar between the bait and the stomach contents of octopus (Figure 6.4.1 and Figure 6.4.2) while DHA/EPA was high, comparatively to any tissue of the octopus.

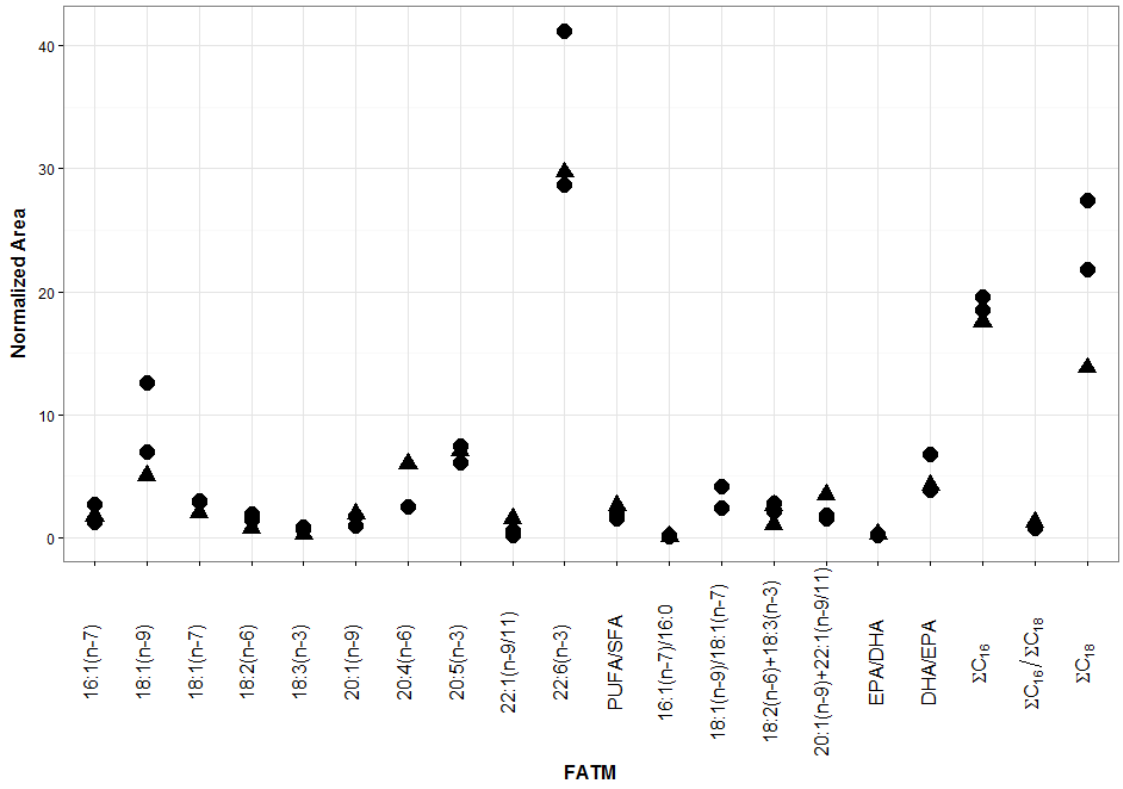

Figure 6.4.2 Fatty Acid Trophic Markers expressed as normalized areas percentage of fish bait used in the *O. vulgaris* fishery: *Trachurus trachurus* (triangles) and *Scomber* sp. (circles).

6.4.2 Stable isotope composition

The analysis was applied to the 32 cephalopods where data was successfully obtained for all tissues. The distance of sampling had no effect in the stable isotope composition. Strong inter-tissue differences ($p<0.0001$) with enriched isotopic ratios, $\delta^{15}N$ and $\delta^{13}C$ in the mantle indicated a change in the recent diet. There were no significant differences in the isotopic values of digestive gland, stomach contents and also the bait.

Carbon isotope ratios had higher variability in the digestive gland than in the mantle or the stomach contents of the octopus with a mean ratio strongly enriched in the mantle that was significantly different ($p<0.0001$) from the bait.

Nitrogen isotope ratios higher variability in the stomach contents than in the bait assumed the stomach did not contain exclusively fish muscle from the bait. These values of the $\delta^{15}N$ ratios showed no significant difference to the mantle of the octopus (Figure 6.4.3 and Figure 6.4.4).

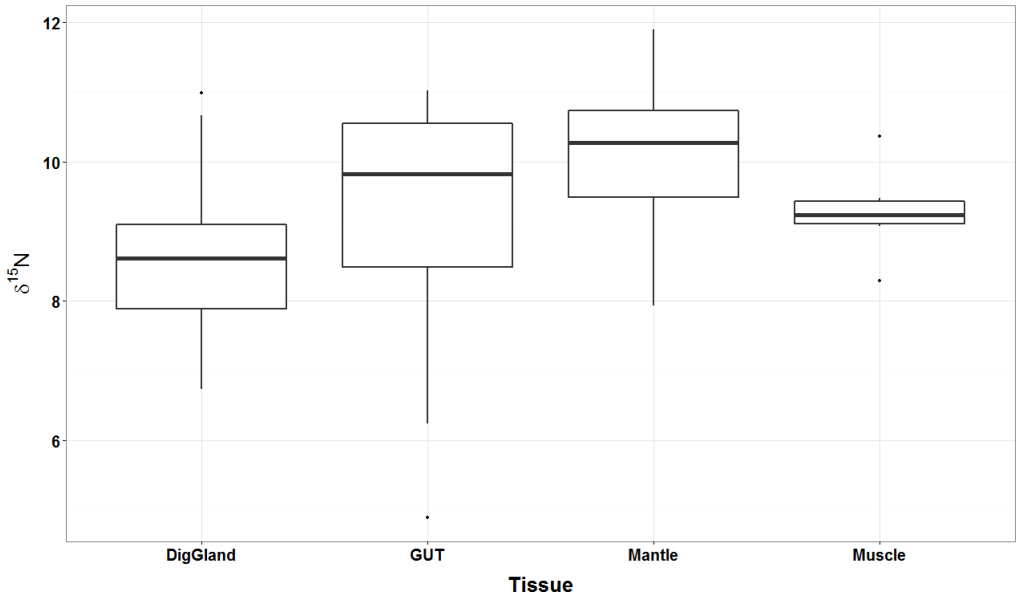

Figure 6.4.3 Boxplots of $\delta^{15}N$ values of digestive gland tissue, stomach contents, mantle tissue of *O. vulgaris* and fish white muscle tissue used as bait in traps (n=6) (e.g. *Trachurus trachurus*, *Scomber* sp.). Statistical differences: $p<0.00001$: Digestive gland-Mantle; Gut[a]-Mantle; n.s. Digestive Gland-Gut[a]; Gut[a]-Muscle[b]; Mantle- Muscle[b]; Digestive Gland-Muscle[b]. [a]same as stomach contents, [b]fish bait muscle.

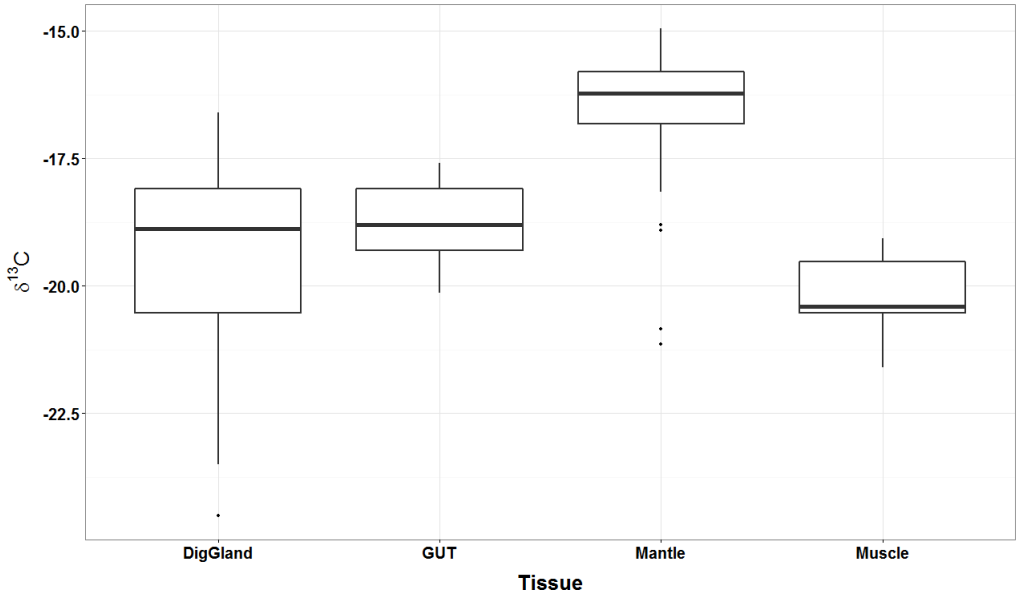

Figure 6.4.4 Boxplots of $\delta^{13}C$ values of digestive gland tissue, gut contents, mantle tissue of *O. vulgaris* and fish white muscle tissue used as bait in traps (n=6) (e.g. *Trachurus trachurus*, *Scomber* sp.). Statistical differences: p<0.00001: Digestive gland-Mantle; Gut[a]-Mantle; Mantle-Muscle[b]; n.s. Digestive Gland-Gut[a]; Gut[a]-Muscle[b]; Digestive Gland-Muscle[b]. [a]same as stomach contents, [b]fish bait muscle.

6.5 Discussion

This study demonstrated that offshore pelagic fish when available is readily incorporated in the coastal benthic trophic web of octopus. These baited traps success is due to the release of chemicals that explore the opportunistic feeding behavior and the constant seek for refuge of the *Octopus vulgaris* (Gillespie et al. 1998, Archdale and Kawamura 2011).

The increasing fishing pressure in the octopus fishery of this region has a consequent increase in the bait amount (Sonderblohm et al. 2014) available for the ecosystem that will increase scavengers and cephalopods paralarvae predators with effects on trophic interactions and ultimately a negative impact in the octopus recruitment. Bait is usually composed of offshore pelagic fish species obtained from bycatches of other commercial fisheries or at the local market (Sonderblohm et al. 2014) and as such, the

ecological impact of harvesting for this specific bait is low. The offshore pelagic fish is an outsource food item and thus, not usual in the diet of this cephalopod (Saila et al. 2002, Sheehan et al. 2008, Cherel et al. 2009, Waddington and Meeuwig 2009, Archdale et al. 2010) and typically is high in DHA and EPA with distinctively greater proportions of DHA (Bandarra et al. 2001, Salma et al. 2016).

The type of bait affect catch rates and other species are used as bait besides fish species (Taborda 2012). The main food source for *O. vulgaris* is crustaceans (Gillespie et al. 1998) whose levels of EPA reach two fold the levels of DHA (Naczk et al. 2004, Stanek et al. 2011) and reach 15% of the bait used in the regional fishery (Taborda 2012). The slower decomposition of live crabs (e.g. *Carcinus maenas*) as bait redirects fishing effort to deployment of higher number of fishing traps opposite to dead fish bait that requires daily replacement (Sonderblohm et al. 2014). The harvesting of these crabs for the octopus fishery in habitats that are environmentally sensitive has a higher ecological impact when the decline in the crab population causes cascading effects to predatory birds (Sheehan et al. 2010, 2012) and prey species (Naczk et al. 2004, Baeta et al. 2006, Archdale and Kawamura 2011).

This study also showed temporal variability of the diet of octopus but reduced the importance of small scale space, due to the differences in trophic markers profiles among tissues but not within tissues.

From the trophic markers analysis it was possible to deduce marine ecosystem as the source of nutrients for octopus (Bouillon et al. 2011) mainly from a wide range of algae. From the tissues of the octopus, the variability in EPA showed a recent change in the origin or composition the energy sources (Connolly 2003a) and the high concentrations of DHA support the predation feeding behavior and the dominance of dinoflagellate at the base of the trophic web, despite a recent raise in the diatom importance deduced by the EPA concentrations in the digestive gland (Dalsgaard et al. 2003). Stomach contents were composed of fish, presumably the bait in the traps, due to similar levels of 18:1(n-9) and DHA and particularly low EPA. Nevertheless, the absolute strength of energetic subsidies by this bait for the trophic web of the octopus

was weak, in the studied time frame. The consumption of unusual prey reflects the opportunistic nature of the feeding behavior of the octopus.

The recent increase of pelagic food sources relative to benthic organisms is sustained by the values of $\delta^{13}C$, which are under -20‰ in marine consumers that derive their carbon from phytoplankton (France 1995).

This study has some limitations worth mentioning. The stomach contents analyzed were composed of full fish and not just fish muscle, whereas the bait was analyzed only for white muscle. Consequently, the fatty acids profiles were different between stomach contents and bait. Despite this, trophic markers are affected by metabolism and it becomes imperative to know metabolic modifications of trophic markers after ingestion instead of concluding on diet solely from the fatty acid composition of tissues (Kelly and Scheibling 2012).

As a benthic predator, the octopus is enmeshed in the benthic food web and the isotopic variation may reflect temporal changes in the sources of organic matter by the opportunistic consumption of prey that forage between the marine benthic environment and habitats where isotopic variation is large, e.g. estuaries (Kirsch et al. 1998b).

The trophic markers in tissues lack the capacity to signal pulse opportunistic intakes but reveal trophic links between habitats with distinct organic sources since organisms will pass on the trophic markers signature up the food web (K. Abrantes et al. 2015). The depleted carbon isotope ratios and the proportions of the fatty acids 18:2(n-6) and 18:3(n-3), sustained an irrelevant uptake of organic terrestrial sources, and since occasional inputs are hardly detected (Dalsgaard et al. 2003), terrestrial matter is rejected as a source of energy for the octopus benthic trophic web even through foraging prey from estuaries.

A possible scenario for the enriched isotopic ratios in the octopus tissues is the predation of benthic consumers that primarily consume seagrass (Melville and Connolly 2005).

The limited ability to move long distances for food or a high degree of site fidelity implies the lower variability of trophic markers from the mantle comes from a higher feeding on local resources and lower predation on foraging species in the larger time frame. In the digestive gland and stomach contents, the higher variability in the values of trophic markers suggests higher consumption of species foraging from habitats with distinct organic sources or at different trophic levels.

The limitation for the interpretation of the $\delta^{15}N$ values is the lack of information on the baselines values for this benthic trophic web (Post 2002, El-Sabaawi et al. 2009). Data on isotopic values from different habitats enables to establish more comprehensive energy connectivity links between habitats, even if through bait.

In summary, this study provides evidence of pulse feeding by *O. vulgaris* for an unusual food source. This trophic event may persist if the supply of outsource species becomes regular and it will consequently reinforce the trophic connections between environments.

7 General Discussion

The present work demonstrates the importance of habitats connectivity in the coastal environment and the sources of primary production for trophic webs. Coastal areas are energetically supplied with organic matter from adjacent habitats, as estuaries, offshore habitats and inland habitats. The relative importance of terrestrial and marine organic matter to coastal aquatic productivity is still an ongoing debate and deserves continuing attention (Kimmerer 2002, Connolly et al. 2009, Kostecki et al. 2010).(Naman et al. 2016, De Cesare et al. 2017). The trophic markers were fundamental to obtain information on the variability of trophic dynamics for fishes in the estuary and the links between the terrestrial and coastal environments and for the cephalopod. These fishery resources have great potential to create links to different habitats and are susceptible to variability in environmental conditions with consequences for productivity.

This thesis applied as trophic markers, fatty acids composition and stable isotopes composition in tissues of consumers from coastal habitats to determine the importance of the different energy sources, spatial and temporal dietary shifts.

Despite the great importance to acknowledge trophodynamics for studies of biodiversity and productivity, few trophic studies in the East coastal of the Algarve applied the combination of fatty acids and stable isotopes as trophic markers to add new dimensions to the knowledge on trophic links and the trophic connectivity between habitats. These techniques provide scientific based information in a fast and effective way on the trophodynamics between species in this multi-habitat approach and the environmental features and anthropogenic activities that influence the dynamics predator prey, with the ultimate goal to predict fisheries productivity in coastal areas (Connolly et al. 2009, Kostecki et al. 2011, Naman et al. 2016).

Certain fatty acids described as trophic markers are specific to primary producers. This feature allows qualitative dietary assessment of the base of the food web of organisms in the benthic environment. Stable isotope analysis is another technique that also

depends on diet source and tissues and supports conclusions on dietary analysis (Dalsgaard et al. 2003, Sherwood and Rose 2005, Petursdottir et al. 2008, Cardona et al. 2015, Giraldo et al. 2016, Espinoza et al. 2017).

The temporal diet shifts were interpreted from the inter tissue differences in the trophic markers composition while spatial diet shifts were interpreted from intra tissues differences (Stowasser et al. 2006, Deudero et al. 2009). The application of fatty acids and stable isotopes was successful to determine the incorporation of terrestrial organic matter into estuarine and coastal fish food webs. The combination of both methods ascertained the low contribution of terrestrial sources for the fish productivity in the estuaries and adjacent offshore areas. Tissues with similar function showed no differences in trophic markers and their simultaneous use in the analyses was ineffective to detect diet shifts and thus, discontinued. Stable isotopes composition is affected by lipidic content (DeNiro and Epstein 1977), which must be extracted to improve the analysis of diet variability.

The estuarine habitat diverges from the adjacent coastal zone in the sources, quantity and quality of nutrients available for primary producers productivity (Schlacher et al. 2009). Despite the continuous input of terrestrial productivity in the estuary, aquatic productivity was still the main energy source for the fish food webs. The combination of biomarkers consolidated conclusions that otherwise would have been erroneous. Separately, the results from each technique would mislead conclusions (De Lange and Van den Brink 2006). Stable isotope analyzed exclusively would amplify the importance of terrestrial matter transported by river flow to the fisheries productivity in estuaries and coastal habitats. Feeding behavior of fish caught in the Guadiana estuary was highly supported by the fatty acid analyses. Energy outsourcing was mostly predominant in the low estuary, as mid estuary and offshore fish preyed on local food sources. In the low estuary, the food sources for fish were supplied by water movement from the ocean or the fish retrained from feeding in the estuary. This way the combination of methods ascertained the low contribution at this time of the terrestrial sources for the fish productivity in the estuaries and adjacent coastal zone. Despite the closeness of the sampling sites within the estuary, there were differences

between the energy sources in the diets of the fish sampled. The movement of individuals or food between closer sites matched the trophic markers composition between closer sites, whilst fish from further sites showed a more pronounced distinction between trophic markers. Typically, even across regions and habitats, sites supplied with different sources of organic material are likely not reflected due to the movement of fish (Brewster et al. 2017).

The connectivity between the estuarine and coastal habitats was not by the input of terrestrial matter as an energetic source in the estuary at this point in time, but as fragile habitats, any environmental changes in the hydrology or anthropogenic activities may affect the quantity and quality of terrestrial organic matter and have cascading effects in habitats connectivity and trophic structure and functioning of the estuarine and coastal communities. Terrestrial organic material is known in other locations to complete energy requirements for the trophic webs of coastal fisheries species and enhance productivity (Connolly et al. 2009). This material source can supply energy to juveniles of several species in the estuarine area of influence like the river plume or may contribute with nutrients for primary producers (Vinagre et al. 2008, Kostecki et al. 2010).

The weak energetic connectivity in this season between terrestrial and aquatic (estuarine and marine) environment can be attributed to open boundaries of the estuary through which fish and food sources move; extreme temporal variability in the freshwater flow of this river and use of this estuary by fishes for other purposes rather than feeding (Loneragan and Bunn 1999, Vinagre et al. 2008, Abrantes and Sheaves 2010). This reinforces the importance of continued studies on the transfer of nutrients between these dynamic coastal food webs.

Evidence of the freshwater flow effects on trophic pathways of estuarine and coastal organisms, mainly fisheries species, will strengthen management procedures for river flow. A broader study, at the population and community level, of estuarine and coastal benthic species will better explain consequences of modifications in trophic interactions in species that use multiple habitats, since one of the main constraints of

this study was the insufficient fish biomass in the estuarine areas for more explicit results.

For the food web of the cephalopods caught in the coastal subtidal of eastern Algarve, a dinoflagellate based food web was established, as well as the reduced contribution of terrestrial organic matter in the recent diet of this cephalopod, according to the typical fatty acid trophic markers from vascular plants. So, for this cephalopod at the pre-adult stage, the land-coastal trophic coupling was unclear. The inter tissue analysis for diet variability in time is debatable due to tissue specific metabolic reactions. Nevertheless, one behavioral aspect of this cephalopod was supported by the analysis of trophic markers: the strong opportunistic feeding that includes unusual prey. Within and among tissues analyses and the feeding behavior of the generalist predator, *O. vulgaris*, towards allochthonous food ensured solid basis for the conclusions on the quickness of diet shift with the changing conditions of the surrounding environment to avoid competition with conspecifics, minimize energetic costs and predation risks (Camprasse et al. 2017). The diet of this cephalopod is invertebrate-based only but the reaction to the variability in food sources demonstrated clearly the opportunistic feeding behavior - this cephalopod readily consumed prey species not available naturally in the benthic habitat, a pelagic fish. It would be interesting to verify if the introduced prey had a higher trophic position than that of the cephalopod and the role in the structure of the trophic web of this type of pelagic prey.

Cephalopods have a high quality fatty acid composition that may be affected by the prey available (Biandolino et al. 2010, Prato et al. 2011, Lourenço et al. 2017). The introduction of allochthonous prey from distinct environments readily assimilated by the octopus may quickly have consequences on the short term for the expected quality in the fatty acid composition of this valuable marine resource. The turnover rates of each tissue and the metabolic effects for the tropic markers are essential to understand and establish so that any diet shifts is determined very accurately (Pinnegar and Polunin 1999, Phillips and Eldridge 2005, Stowasser et al. 2006).

The variables that contributed to the recent increase in the catches of this cephalopod are urgent to identify. An increase in fishing effort unaccompanied by an increase in

productivity will undoubtedly lead to a population decline. Nevertheless, if the population increased due to environmental driven conditions, as supply of food or increase in paralarvae survival due to changes in habitat conditions or a decrease in predators, it is advisable to understand if pulse events affect short term productivity for this multi-habitat species that is extremely sensitive to environmental conditions (Raimundo et al. 2017). An example of a pulse event is any activity that increases the river flow or the quantity or quality of terrestrial organic matter with consequences on the energy supply to this species and productivity. At this point in time and space, the Guadiana river outflow role had a minor role for the *O. vulgaris* diet and thus, for a short-term productivity.

Ideally, all components of the trophic web should be included in the analysis, from inorganic nutrients to phytoplankton, zooplankton, primary consumers and higher trophic levels and inclusively, humans. Marine species are high in contents of n-3 PUFA, especially EPA 20:5(n-3) and DHA 22:6(n-3) that are long known for their nutritional benefits in the human diet. Humans are usually not included in marine trophic studies despite being the ultimate predator and the agent for fluctuations in fisheries targets.

Analyses of baseline organisms will assess spatial variability of primary producers on a much reduced time scale and accurately estimate the trophic position of the food web components. Bivalves are an example of a key baseline organism for trophic web studies for their wide availability and reduced mobility. Attention should always be given to inter tissue variability in trophic markers even in baseline organisms due to metabolic reactions (Redmond et al. 2010).

Trophic markers showed advanced information on the temporal and spatial variability on the energetic sources of predators in coastal environments, highly explored by fisheries activities. However, physiological effects on the biomarkers must be acknowledged to discern from trophic interactions and this is only achieved with controlled feeding studies for the species under study (Stowasser et al. 2006). Another important aspect to consider in the studies from the trophic markers in the estuary and adjacent coastal areas are the pooling of samples and sample sizes, as well as the

chosen species. The pooling of samples for higher numbers of fish of the same species and size caught in the same sampling occasion was performed due to conditions in the analytical process and available resources, while the small number of samples in the estuary was a result from environmental conditions despite the high fishing effort. The impossibility to choose a target species in the estuary and coastal study was a consequence of the reduced fish abundance and high variability among sampling sites.

Nowadays, innovative techniques as compound specific isotope analyses overcome fatty acids and stable isotopes. Therefore trophic interactions and foraging behavior that link habitats can be further understood, including details on the components of organic matter assimliated (Logan and Lutcavage 2013, Liénart et al. 2016, Mohan et al. 2016, De Cesare et al. 2017). This technique is especially effective when food sources have similar isotopic and fatty acid composition and when there is an unknown gap of food sources between the base of the food web and the consumer (Gladyshev et al. 2012, Logan and Lutcavage 2013, Steffan et al. 2013). This technique included in future trophic studies will provide unquestionable results on trophic behaviors of consumers, ecosystem impacts on trophic webs and ultimately, on coastal productivity and fisheries resources. The metal bioaccumulation along with trophic markers will also give new insights to trophic ecology of marine species and exposure to metallic inputs (Le Croizier et al. 2016).

In this study, compound specific isotope analyses could easily determine the effect of modification of food sources along the food web on higher trophic levels and determine if allochthonous food are an effective trophic subsidy to promote productivity (Riera et al. 1999, Abrantes and Sheaves 2009). Aminoacids-compound specific isotopic analysis (AA-CSIA) greatly explain trophodynamics and trophic position (Steffan et al. 2013, Gutiérrez-Rodríguez et al. 2014) and other molecular methods specify trophic selectivity of paralarvae and suspension feeders that quickly signal any change in diet (Roura et al. 2012, Maloy et al. 2012).

Another important aspect of concern is the increasing presence of organic matter derived from fish farm in these coastal species. This is an example of an anthropogenic activity with consequences for the organic load in the sediment that can expand up the

trophic web. Future studies can address this matter with the same techniques to monitor the impact on benthic communities and fisheries productivity of this increasing industry (Mayor et al. 2017).

This study adds awareness to the multitude of scenarios yet to be investigated and emphasizes the effectiveness of fatty acids and stable isotopes as trophic markers in consumers to detect variability at the base of the food the web and diet shifts and habitat linkages by trophic pathways. The incorporation of trophic markers and the temporal variability associated to the food assimilation is evidence for diet shifts with several environmental conditions to be examined. From climate change, modification in oceanic currents and hydrological cycle to anthropogenic activities (wastewater, terrestrial matter, seafloor dredging), what are the events that scale up the trophic web to productivity?

Author comments:

Upcoming publications from this thesis are pending on the revisions of the co-authors.

The following are the predetermined titles:

- Biomarker based trophic structure of fish across an estuary;
- The role of pelagic fish as prey for *Octopus vulgaris* in the benthic food web assessed with stable isotopes and fatty acids;
- Trophic markers reveals diet shifts in *Octopus vulgaris*;
- Fatty acids and stable isotopes as tools to detect habitat trophic connectivity.

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